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The Bacterial Ecology and Function from a Sub-Surface Critical Zone

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B.Sc. Marine Biology (Hons)

M.Sc. Biological Diversity

**A Thesis Submitted for the Degree of Doctor
of Philosophy**

Molecular Ecology

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Hydrology, Wallingford, UK

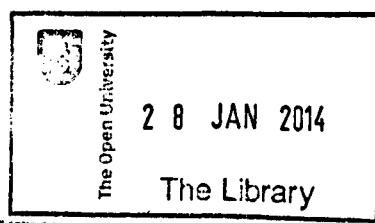
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Declaration

The research described herein was funded jointly and conducted at The Open University (Geomicrobiology group) and the NERC Centre for Ecology and Hydrology (Wallingford; Molecular and Microbial Ecology Group). All of the research carried out in this thesis is my own original research, with the following exceptions:

- 454 next generation sequencing (several chapters). This was conducted by a technical staff of a commercial bioinformatics company (Research and Testing Laboratory, Lubbock, Texas). All subsequent bioinformatic analysis was conducted by myself.
- XRF analysis (Chapter II) – sample preparation was conducted by myself but final analysis was conducted by John Watson (Open University, UK).
- Measurement of solute liberated analytes (Chapter IV) – This was performed by technical staff at the aquatic chemistry laboratory in NERC CEH (Wallingford, UK)
- Phospholipid fatty acid analysis (supplementary data) – this was performed at the University of Bristol by technical staff. Awarded under grant – NERC LSMSFBRIS004.
- Sample site determination and collection was conducted jointly by myself and Prof. Charles S. Cockell.



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- Mostly I would like to thank my wife and family for being so patient during the past four years.
 - Oh I suppose the dog should get a 'look in' to for keeping my feet warm under the desk during the write up phase – “Who’s a good pig Chester”.

Abstract

The rock-soil interface (critical zone) is where a variety of important earth system processes occur, such as the sequestration of CO₂ and pedogenesis from rock weathering. This zone is an important sub-surface region of microbial activity in rocky environments because bedrock hydrolyses providing nutrients and cations (i.e. Ca²⁺ Mg²⁺, Na⁺) otherwise unavailable to flora and fauna. Yet, the diversity and role of microorganisms in weathering at the critical zone is not well understood.

This thesis examines microbial communities in vegetated and unvegetated critical zones near Skorradalur Lake, Iceland. A suite of analyses were carried out to determine the environmental conditions, diversity, function and trophic survival strategy employed by bacteria at the sub-surface critical zone.

Molecular analysis of the bacterial 16S rRNA gene indicates that the presence of plants at the soil surface influences the bacterial diversity and composition. Cultivation of microorganisms produced several bacterial isolates; most of which were capable of mineral phosphate solubilisation. However, isolate growth rates and copper tolerance show most isolates inhabit areas of sub-optimal growth potential.

Environmental factors such as temperature and pH may influence bacterial diversity, although the presence of organics may override these other influences. The trophic conditions at sites without higher plants may have a role in bacterial weathering as the availability of organics has resulted in a diverse heterotrophic community capable of utilizing bacterial necromass as a carbon source rather than plant derived carbon.

This work has shown for the first time the bacterial diversity of the sub-surface critical zone in this region. Moreover, this bacterial diversity is influenced by plants and the potentially associated organics. Areas devoid of plants harboured a diverse heterotrophic bacterial community that employ weathering as a generalist function. Experiments show that bacteria increase the rate of phosphate weathering, although it may be a generalist function rather than specific to any individual taxon.

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Abbreviations and acronyms

(Q) RT-PCR	Quantitative Real Time Polymerase Chain Reaction
6FAM	6-Carboxyfluorescein
Alk	Alkalinity
ANOSIM	Analysis of Similarities
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
bTEFAP	Bacterial Tag-Encoded FLX Amplicon Pyrosequencing
CALS	College of Agriculture and Life Sciences
CCA	Canonical Correspondence Analysis
CEH	Centre for Ecology and Hydrology
CTAB	Hexadecyltrimethylammonium Bromide
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DTPA	Diethylenetriaminepentaacetic Acid
EDTA	Ethylenediaminetetraacetic acid
ENVFIT	Environmental Fit Correlation
FAME	Fatty Acid Methyl Ester
Fe _t	Total Iron
GC	The DNA Bases Guanine and Cytosine
GC/C/IRMS	Gas Chromatography Combustion Isotope Ratio Mass Spectrometry
GC/MC	Gas Chromatography Mass Spectrometry
GDH	Glucose Dehydrogenase
GL	Glycolipid
HPLC	High Performance Liquid Chromatography
ICP	Inductively Coupled Plasma
ICP-AES	ICP Atomic Emission Spectroscopy
ICP-OES	ICP Optical Emission Spectroscopy
ITOL	Interactive Tree of Life
LB	Lysogeny Broth
LOI	Loss On Ignition
LMS	Lichen and Moss Covered Site
LWMOA	Low Weight Molecular Organic Acid
MEGA	Molecular Evolutionary Genetics Analysis
MPS	Mineral Phosphate Solubilisation
n.t.	Nucleotides Base
NGS	Next Generation Sequencing
NIST	National Institute of Standards and Technology
NMDS	Non-Metric Multidimensional Scaling
OD	Optical Density
OTU	Operational Taxonomic Unit
PAR	Photosynthetically Active Radiation
PBS	Phosphate Buffered Saline
PCA	Principles Component Analysis

PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PL	Phospholipid
PLFA	PL Fatty Acid Analysis
PQQ	Pyrroloquinoline Quinone
QIIME	Quantitative Insights Into Microbial Ecology
RDP	Ribosomal Database Project
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SIMPER	Analysis of Dissimilarity
SIP	Stable Isotope Probing
SL	Simple Lipid
SOC	Soil Organic Carbon
SOM	Soil Organic Matter
SRP	Soluble Reactive Phosphorus
<i>Taq</i>	<i>Taq</i> Polymerase
TCP	Tri-Calcium Phosphate
TCS	Tree Covered Site
TLE	Total Lipid Extraction
tRF	Terminal Restriction Fragment
tRFLP	tRF Length Polymorphism
UV	Ultraviolet
XRF	X-Ray Fluorescence

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Chapter I

Bacterial function at the sub-surface critical zone

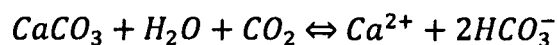
1. General introduction

1.1. The importance of weathering

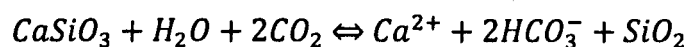
The Earth's geological record indicates that climate can vary substantially over many millennia (Mayewski et al., 2004, Moreno, 2004, Rind & Overpeck, 1993), but has yet to succumb to catastrophic 'Snowball Earth' or runaway greenhouse scenarios observed on other planetary bodies (Caldeira & Kasting, 1992, Sleep et al., 1989). Global feedback systems are thought to be responsible for this buffering against significant global scale climatic events, by controlling the Earth's atmospheric greenhouse gas composition, for example the cloud seeding properties of the coccolithophore *Emiliania huxleyi*. DMS production during coccolith production can aid in cloud formation and result in an increased albedo effect, ultimately lowering the temperature of the water column (Kump, 1996). Another such stabilizing process is the sequestration of carbon from the atmosphere, enhanced by the weathering of bedrock at the sub-surface critical zone (the rock-soil interface at which bedrock denudates).

Through the weathering of several minerals, carbon dioxide (CO₂) is drawn down from the atmosphere as a consequence of the chemical reaction between substrate (typically carbonate or silicate) and water (H₂O) as dictated by reactions observed in the following equations;

(1.1) Weathering reaction of a carbonate mineral



(1.2) Weathering reaction of a silicate mineral



Not all of the CO_2 used in these reactions is sequestered and stored; the volume of carbon stored is highly dependent on the particular minerals undergoing weathering (Berner et al., 1983, Garrels & Mackenzie, 1971) and may result in CO_2 that has been sequestered being stored within the Earth's oceans, potentially for millions of years (Berner, 1999, Urey & Korff, 1952). As indicated, two common minerals that weather easily are carbonate and silicate. In both cases, the weathering reactions result in a gross sequestration of CO_2 from the atmosphere. However CO_2 sequestration during weathering of silicates is double that of carbonate (Berner, 1992).

The weathering chemical reaction consists of atmospheric CO_2 and H_2O , normally from precipitation events, coming into contact with bedrock. Precipitation events are known to dissolve atmospheric CO_2 , forming a weak carbonic acid and this acid can dissolve minerals present in the bedrock. In the case of carbonate weathering, calcium (Ca^{2+}) and bicarbonate (HCO_3^-) are the net liberated products (Equation 1.1). However for silicate weathering, the net products of the reaction are Ca^{2+} , 2HCO_3^- and silica (SiO_2 ; Equation 1.2). The release of SiO_2 can be detected in soils following weathering, through a simple molybdenum blue assay, and is frequently used as a proxy to quantify the rates of silicate weathering (Navarre-Sitchler & Brantley, 2007).

The HCO_3^- resulting from the weathering reaction can be transported from the critical zone via local water systems (Banfield & Nealson, 1997). The end product of this transportation is deposition of the HCO_3^- into the world's oceans in the form of carbonate via the reversal of Equation 1.1. As this reaction reversal releases CO_2 to the environment, there is no net storage of carbon during carbonate weathering, but a net storage of CO_2 does occur from bicarbonate originating from

silicate weathering (reversal of equation 1.2). This net sequestration from the weathering of silicates has been reported to store between 1.5 and 2.6×10^8 tons $C\ y^{-1}$ from the atmosphere (Arthur, 2000, Hilley & Porder, 2008, Varekamp et al., 1992).

While carbonate rocks, such as dolomite, aragonite and calcite are common at the critical zone, the lack of net carbon sequestration has increased scientific interest for examining alternative substrates involved in critical zone weathering (see Dessert et al., 2003 for a review). In particular, basalt and basalt glass have been intensively studied (Cockell et al., 2009b, Furnes, 1975, Mason et al., 2007, Navarre-Sitchler & Brantley, 2007, Santelli et al., 2009, Thorseth et al., 1995), as they weather readily in both field and laboratory conditions. For example, alteration textures believed to be bacterial in origin, have been visualised using scanning electron microscopy of basalt glass structures (Cockell et al., 2009a, Thorseth et al., 1992). Silicate weathering also results in the liberation of several bio-essential elements, such as Mg, Fe and Si. Weathering can be measured through the liberation of these metals and other compounds, and many studies have concentrated on the fluxes of these solutes through the local freshwater systems (Gomez-Alvarez et al., 2007, Moulton et al., 2000, Suchet & Probst, 1995, Turner et al., 2010).

Although weathering is based upon the chemical reactions described here, their origins in the environment can be varied. Discussed here are some of the processes that can catalyse and/or enhance these reactions at the sub-surface critical zone.

1.1.2. Weathering processes

Weathering is a slow process that ultimately leads to pedogenesis (soil formation). The on-going denudation of bedrock results in ever smaller particles, ranging in size from boulders (> 630 mm) to colloidal particles (≤ 0.001 mm). This process is generally catalysed in three ways: via physical, chemical and biological mechanisms.

One well-known process of physical weathering is freeze-thaw disaggregation, characterised by the expansion and contraction of the anisotropical bedrock, resulting in substrate fractures (Siegesmund et al., 2000). This can be further enhanced by the inclusion (and expansion) of H_2O in the cracks and pores of the substrate during these freeze-thaw cycles. Other forms of physical weathering occur, though these are less well-known. For example, physical erosion of rock and soil can weather bedrock, but additionally this alters the pressures acting on submerged bedrock. Whilst erosion alters the mass overlaying the bedrock, massive pressures can be relieved and result in fracturing of the bedrock surface layers (Camuffo, 1995).

Chemical weathering is a commonly studied mechanism as the solutes liberated during weathering are easily measured and reported (see Lasaga et al., 1994 for a review). In brief, chemical weathering is driven and controlled by several factors such as water-rock interaction, pH, ionic strength and dissolution rates, which all potentially co-vary and co-contribute toward weathering of substrates. Acid-induced weathering (low pH solutions dissolving the structure of bedrock) can occur through the release of organic acids produced by biota (Rose, 1957) or through acid rain (Johnson et al., 1981). Indeed, acidic precipitation has been shown to have an impact on the weathering rates of silicate structures in western Iceland, for example the mean pH of Icelandic precipitation ($pH = 5.4 \pm 0.46$) was

observed as 0.2 pH units below the classification limits of acidic precipitation (pH 5.6), although this was marginal. In addition to direct weathering from this acidic-precipitation, this lowered the pH of the local water systems, enhancing the release of Ca, Si and Al from local basalts (Gislason et al., 1996).

Biological weathering can best be described as a combination of bio-chemical and bio-physical methods. Individual organisms are capable of exerting a physical stress on to the bedrock while exuding organic acids and/or chelating agents capable of altering the environmental conditions; effectively enhancing the efficacy of chemical and physical weathering. It is for this reason that biotic influences on weathering have received a great deal of attention in recent years. In brief, the influence of bacteria to weather minerals and rocks has been observed, as has the subsequent release of cations to the environment (Abdulla, 2009, Mailloux et al., 2009, Smeaton, 2009, Štyriaková et al., 2012, Wilson et al., 2008). A more complex system that has also been investigated is the interaction between bacterial weathering and the rhizosphere, where several key bacteria have been shown to be influenced by the floral stands they inhabit (Calvaruso et al., 2006, Calvaruso et al., 2010, Puente et al., 2004, Uroz et al., 2007, Uroz et al., 2009b, Uroz et al., 2011a, Uroz et al., 2011b). However, the effect of biota at the sub-surface critical zone has received little attention to date and is therefore a focus of this study.

1.1.3. Pedogenesis

One eventual outcome of the weathering of bedrock is enhanced pedogenesis, (Figure 1.1), the process by which bedrock evolves into a soil structure, resulting in a soil layer covering the bedrock. Initial pedogenesis of soils is followed by deepening of the soil profile caused by organic deposition and decomposition

and/or continued bedrock fracturing and weathering. Furthermore, sedimentary deposition can occur from remote locations as particulate matter can be transported via water systems and atmospheric deposition, promoting enhancement of the soil profile. Pedogenesis can occur through several processes. In brief, biotic driven pedogenesis results from deposition of organic matter which gradually degrades to form a soil horizon. This process is a top-down mechanism resulting in newer soils at the surface with older soils being deeper and retaining many of the biotic signatures and nutrients of their creation. In contrast, bottom-up weathering induced pedogenesis occurs as bedrock denudates and results in newer soils at depth with older oligotrophic soils nearer the surface. The processes involved in each are described in detail by Almond and Tonkin (1999). The composition of any soil profile will depend on the rate that each process occurs. For example, predominately bottom up pedogenesis will result in a greater proportion of oligotrophic areas within the soil profile, whereas predominately top down pedogenesis will result in a small oligotrophic zone at the sub-surface with copiotrophic horizons being dominant throughout the soil profile. As both of these processes will occur in tandem the determination of whether a soil is oligotrophic or copiotrophic will be largely influenced by the efficiency of each process. Warmer forest soils rich in flora and fauna will in all likelihood have a greater rate of pedogenesis from the surface, than the subsurface, whereas barren landscapes such as Iceland will have lower biotic rates of pedogenesis and may rely more on bottom-up processes for pedogenesis.

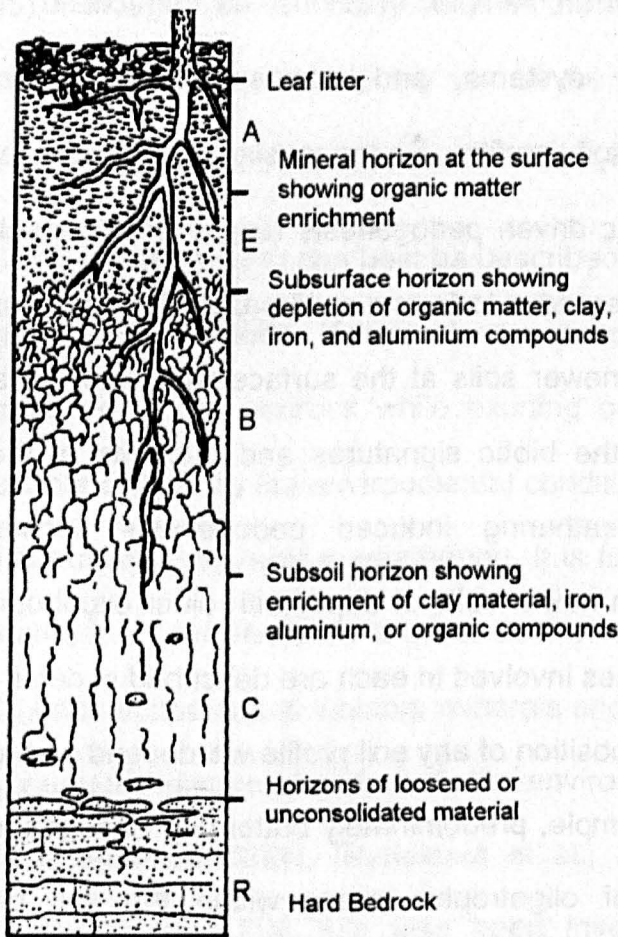


Figure 1.1: A representative soil profile indicating the soil horizons from the Organic surface layer, topsoil (A), subsoil (E), saprolite (B) to the sub-surface critical zone (C) with lowest layer representing bedrock (R). Figure reproduced from CALS (Manual). Soils classified as defined by (Avery, 2006).

As weathering induced pedogenesis occurs, horizonation of the soil profile follows with older minerally-diverse soils at the surface and newer, less weathered, soils located nearer the bedrock at the base of the soil profile (Almond & Tonkin, 1999, Johnson & Watson-Stegner, 1987). As further deepening and weathering occurs, there is a perceived net migration of bioavailable nutrients, minerals and metals toward the surface. However, the overall concentrations in soil horizons remains static, whereas the continuing denudation of rock fragments and soils expose greater surface areas at the shallower horizons, resulting in this perceived enhancement of the rate of mineral mobilization and nutrient concentrations towards the surface (Rumpel et al., 2002).

The organic horizons at the soil surface are generally rich in biota, minerals and organic carbon. The same is true of the mineral topsoil horizon (Figure 1.1; A), however concentrations of bioavailable mineral nutrients and carbon decrease with increasing soil depth, as they are locked in a mineral state unobtainable to most biota. The subsoil and saprolite horizons (Figure 1.1; E and B respectively) offer relatively poor nutrient pools and in the critical zone (Figure 1.1; C) the conditions for life are very limiting with considerably lower organic matter and fewer available minerals and nutrients.

1.1.4 Soil biota

Throughout all soil horizons, there are habitable environments containing a large number of organisms present that can also play a role in the weathering of soils and unweathered rock fragments. Nearer the soil surface (Figure 1.1; A) there are rich carbon pools, resulting from the decay of organic matter, which can sustain macrofauna, including burrowing organisms such as worms, ants and termites that are able to homogenize the soils and redistribute the available nutrients between the soil horizons (Van Breemen & Buurman, 2002). This activity can also exert physical stresses onto the substrate that result in enhanced weathering (Yoo et al., 2005).

Many soil profiles typically include rhizospheric areas, generally located in horizons (E and B), which are relatively poor in organics and nutrients when compared to topsoil. However, plants are capable of sequestering nutrients, tannins and carbon and transporting these through the root system from the surface to these nutrient deprived horizons (Farrar et al., 2003, Hinsinger, 2001). This seeds the rhizosphere, helping to increase biological diversity in this horizon (Garbeva et al., 2004, Zak et al., 2003). These, often extensive, root systems are

also known to increase denudation rates of basalts during weathering; Cochran and Berner (1996) argue that roots are capable of growing in between mineral fragments and cracks in bedrock, exuding low molecular weight organic acids and chelating agents that are capable of penetrating bedrock several millimetres below rock surface to acquire mineral nutrients. The small diameters of fine roots and root tips permit weathering in small cracks and pores of bedrock and rock fragments, similar to those observed at the critical zone (Cochran & Berner, 1996). It is at these small scales that microbiologists have taken an interest in the extent to which microbial activity plays a role in weathering (Berthelin, 1988, Fisk et al., 1998).

1.2. The role of microbes in weathering

1.2.1. Fungal weathering

Microbes are a general description for microscopic members of the phylogenetic domains Bacteria, Archaea and Eukarya. The latter domain contains microscopic members such as the fungi, algae and protists. There is a well-known relationship between plants and fungi in that the presence of plant roots increases the presence of mycorrhizal hyphae in soils (Andrews et al., 2010, Bonneville et al., 2009, Rambelli, 1973). This relationship is symbiotic and is beneficial to both organisms; while plants provide organics for fungi, there is also evidence suggesting that fungi are capable of weathering minerals and liberating insoluble nutrients previously unavailable to the root system of the plant (Barea et al., 2002, Derry, 2006, Kim et al., 1997). In certain forest soils, as much as 90% of roots tips were recorded as being enveloped in ectomycorrhizal fungi (Ek, 1997) and the majority of nutrients taken up by the roots passed through the fungi. In return, the fungi received c. 25% of the carbon fixed by the plant through photosynthesis

(Högberg & Högberg, 2002). However, Jones et al. (2009) explain that this transfer of carbon may be bi-directional, depending on the environmental conditions; as carbon can be sequestered by the plants from the rhizosphere as well as deposited.

The efficacy of fungi to weathering is thought to be significant and the appearance of fungi on land during the late Proterozoic period is suggested as a causal factor in the increase of terrestrial eukaryotic organisms. Fungi-enhanced weathering is believed to have sequestered sufficient carbon to aid in the generation of an oxygen-rich environment, resulting in further improvement in growth conditions required by *Eukarya* (Derry, 2006).

Fungal weathering has also received considerable attention through the study of lichens; a symbiosis between fungi and algae/cyanobacteria. Piervittori et al. (1994) provides a bibliography of lichen associated deterioration of stonework from studies dating back as far as 1825. For example, weathering by lichens has been recorded as the deterioration of statues and stonework directly beneath lichen encrustations (Adamo & Violante, 2000, Danin et al., 1982). However, further research has shown that lichen encrustation can also enhance the weathering of environmental rock structures and subsequently affect the carbonate-silicate cycle (Brady et al., 1999, Matthews & Owen, 2008); these lichen encrustations may enhance environmental rock weathering by up to two orders of magnitude (Matthews & Owen, 2008). One reason for this substantial weathering ability could be that the hyphae of the lichen can reach up to 12 mm below the surface of the rock to which the lichen inhabits, permitting deeper penetration of chelating agents and organic acids (Bjelland & Ekman, 2005). However, this enhanced weathering may be an over estimation of lichen weathering capability; Vingiani et al. (2013)

report that chemical signatures, such as increased Si, used to infer weathering may be an accumulation of particulate matter within the lichen foliage that originates from the environment, rather than directly from the rock surface.

1.2.2. Weathering by phototrophs

Excluding lichens, the study of phototrophs in weathering has traditionally concentrated on *Cyanobacteria*, often using calcium carbonate structures as a substrate (Danin et al., 1982, Ortega-Calvo et al., 1995, Ortega-Calvo et al., 1991, Saiz-Jimenez, 1999). The weathering activity of this taxon on carbonate substrates has also been observed as a potential threat to the deterioration of historical stonework and statues. Crispin and Gaylarde (2005) report that biofilms consisting of *Cyanobacteria* are weathering valuable historical structures and that the need to identify the weathering agents through non-culturable techniques is growing. Furthermore, the weathering ability of this taxon has been shown to be widespread amongst several rock types. This taxon has been associated with weathering of silicate structures from volcanic environments (Olsson-Francis et al., 2012) and can be found c. 4 mm beneath the surface of arid sandstone structures (Büdel et al., 2004), where they have been associated with the alteration of the rock structures in both cases.

Cyanobacteria are not the only potentially phototrophic bacteria capable of weathering; using scanning electron microscopy (SEM), Coombes et al. (2011) showed that generalist microbial colonizers from the photic inter-tidal zone can enhance weathering of carbonate substrates. The use of SEM permitted textural alteration of experimental substrate discs to be visualised. Although, in a subsequent study, they also present evidence of biological protection of the carbonate substrate from weathering through macroalgae coverage (Coombes et

al., 2012). In this case, the presence of macroalgae is thought to protect the substrates from the physical stresses of temperature fluctuations and re-wetting events.

1.2.3. Archaeal weathering

Archaea are thought to be ubiquitous throughout the biosphere at abundances nearing 1×10^5 cells per mL in aquatic environments (DeLong & Pace, 2001) and 3×10^7 in soil ecosystems (Timonen & Bomberg, 2009). However, studies and discussions of microbial weathering generally make sweeping reference to bacteria and archaea as a single group. For example, several studies that refer to microbial weathering discuss the potential for Bacteria and Archaea to weather rock substrates, however there is no data presented to differentiate between these two kingdoms and their roles therein are not defined (Banfield et al., 1999, Brantley et al., 2011, Skidmore et al., 2005). This said, an *in-vitro* examination of microbially enhanced weathering was able to provide evidence indicating that the archaeon *Sulfolobus metallicus* was capable of enhancing the weathering of marcasite at extreme temperatures (Wang et al., 2007a). Given their distribution and the large number of extremophilic organisms within the Archaea, it is possible that the role of archaea in geo-biological weathering is underreported. Hence, significant further investigation, outside the original aims of this study, is required to fully appreciate the role of Archaeal species in the substrate weathering processes of extreme environments.

1.2.4. Bacterial weathering

The role of bacteria in substrate weathering has begun to receive substantial attention (Calvaruso et al., 2006, Cockell et al., 2009a, Cockell et al., 2009b,

Fujimura et al., 2012, Hamdali et al., 2012, Kelly et al., 2010, Mehta & Nautiyal, 2001, Štyriaková et al., 2012). As discussed, initial studies of bacterially enhanced weathering used SEM to look for textural alterations in a substrate (Thorseth et al., 1995). This approach offered qualitative evidence that bacteria are actively involved in weathering but elucidating the mechanisms and biogeochemical processes involved have proven to be a much harder task. However, the role of bacterial species in weathering is now widely recognised (Barker et al., 1998, Collignon et al., 2011, Herrera et al., 2008, Lepleux et al., 2012, Sasaki et al., 1998, Štyriaková et al., 2012, Uroz et al., 2009a).

Table 1.1: A description of the methods that are involved in the weathering of mineral substrates by microbial processes.

Mechanism	Method of weathering	Reference
Acidolysis	The lowering of the pH surrounding individual cells through the release of protons from metabolite production.	(Balland-Bolou-Bi & Poszwa, 2012)
Siderophore chelation	Making Fe ions available to biota through the solubilisation of Fe from minerals	(Ahmed, 2013)
Oxidoreduction	The aerobic oxidation of mineral sulphides to individual metal sulphates. This is specific to the metal compound within the substrate.	(Mapelli et al., 2012)

The mechanisms of bacterial weathering (Table 1.1) are beginning to be addressed (see Uroz et al., 2009a for a review). For example, the initial bacterial colonizers of fresh andosols play a vital role in liberating phosphorus (P) from minerals such as apatite in order to permit the subsequent colonization of vascular plants. This behaviour was seen in *Acidithiobacillus ferrooxidans*, which is able to metabolize phosphonates that are not normally regarded as bio-available (Vera et

al., 2008). Richardson (2001) explored the concept of inoculating soils with bacteria capable of metabolizing both organic and inorganic P making it available for plant uptake, with the aim of converting nutrient-poor andosols into fertile fields. This concept is still a working theory and poses many questions as to which organisms would be best suited to different soil types, however even young andosols, although unreceptive, are capable of hosting highly diverse microbial communities. Nüsslein and Tiedje (1998) describe a 200 year-old andosol in Hawaii with extremely high microbial diversity; they discovered that the soil biomass was dominated by *Pseudomonas*, *Rhizobium-Agrobacterium* and *Rhodospirillum* species, which are all members of bacterial classes linked to the weathering process (Uroz et al., 2009a).

1.3. Bacteria and their role at the critical zone

The critical zone is a key region in which weathering occurs. It can be defined as an area of highly complex and numerous bio-geochemical processes where energy and matter are exchanged across the hydrosphere, lithosphere and atmosphere (Akob & Küsel, 2011, Chorover et al., 2007). While the near-surface soil critical zone is relatively well studied, little is known regarding the soil sub-surface (below, primary photic influence) critical zone or the biological inhabitants that reside there (Akob & Küsel, 2011).

The diversity of soil bacterial communities has been well studied and it is clear that a diverse community inhabits these soil ecosystems (Buyer & Sasser, 2012, Chu et al., 2010). These communities are susceptible to environmental influences that affect this diversity, for example plants have been shown to significantly alter bacterial communities (García-Salamanca et al., 2012, Palaniappan et al., 2010, Thomson et al., 2010). In addition, environmental gradients can also influence the

bacteria community composition, for example pH has been seen to have an effect on the landscape ecology of bacterial diversity (Griffiths et al., 2011, Rousk et al., 2010a, Rousk et al., 2010b). This sustained level of research on the soil bacterial community, has now permitted a detailed understanding of the functional role that these bacteria may have on weathering (Uroz et al., 2011b) and the carbon cycle in general (Nielsen et al., 2011).

The activity of microbes in sub-surface soils may be of great importance in global geochemical cycles. Microbes are believed to play a role in nutrient turnover 10 times greater than that of flora (Whitman et al., 1998). For example, the microbes in soil are thought to be responsible for substantial production and consumption of several soil trace gases such as methane (CH_4), hydrogen (H_2), carbon monoxide (CO), carbonyl sulphide (OCS), nitrous oxide (N_2O) and nitric oxide (NO ; see Conrad, 1996 for a review). In brief, an example is the methylotrophic activity of many soil organisms that releases CO_2 to the atmosphere. Methane is initially converted to methanol by the enzyme methane monooxygenase, then subsequently to formaldehyde by the enzyme methanol dehydrogenase then finally to a readily metabolised form of formic acid (McDonald et al., 2005, Seghers et al., 2006). Conversely, the sequestration of CO_2 in soils can occur through microbial autotrophic behaviours (Tate, 1995), although not all such metabolic redox reactions are possible in all soils. For example, the reduction of bicarbonate to acetate is a metabolically unfavourable reaction, as it is kinetically limited (Seitz et al., 1990). This thermodynamic protection of bicarbonate permits its persistence following weathering reactions at the critical zone.

Microbes are an important inhabitant of the critical zone. The microbial utilization of limited soil organic carbon (SOC) in the deeper soil horizons has a stabilizing

effect on the soil and the SOC pool in general (Fontaine et al., 2007). Moreover, alterations in the activity and carbon availability within the soil microbial communities, have the potential to liberate significant quantities of CO₂ from soil through carbon priming (Carney et al., 2007). This pool of carbon in soils also drives, to a significant degree, the diversity of the microbial population (Lipson et al., 2006) and there is evidence that soil microbial diversity can have globally significant influence on terrestrial SOC pools as c.80% of terrestrial carbon is stored in these ecosystems (Nielsen et al., 2011). Furthermore, Nielsen et al. (2011) infer that the functional traits of these soil organisms rather than their taxonomy may have more bearing on SOC stability, accentuating the importance of functional redundancy in the microbial community.

The functional role of bacteria at the critical zone is of particular interest. Aside from the contribution to SOC stability, they also play a crucial role in the liberation of essential nutrients and minerals from geological substrate (Barker et al., 1998, Herrera et al., 2008, Štyriaková et al., 2012). The functional role of bacteria is important both at the primary weathering stages as well as during the secondary metabolism of the released solutes. A widely studied method that bacteria, in particular, employ to weather silicates is the excretion of organic acids. Gluconic, lactic, acetic, fumaric and malonic acid are a few of the many organic acids produced by a range of bacteria capable of being cultivated (Rose, 1957), which may enhance the mineral phosphate solubilisation of bedrocks; such as that found to occur in silicate materials. This can be assessed by providing bacteria with a substrate of insoluble mineral-state phosphate such as hydroxyapatite or tri-calcium phosphate and measuring the dissolution of the mineral (Hamdali et al., 2012, Jorquera et al., 2008, Mehta & Nautiyal, 2001, Perez et al., 2007, Rodríguez

et al., 2000). Given the diversity of soil bacteria, the number of the organic acids they can potentially produce is also likely to be high.

The ability of bacteria at the critical zone to liberate a large number of mineral elements from bedrock has permitted the development of a diverse bacterial community at the deep sub-surface (Akob & Küsel, 2011). The variety of geological materials available for use as an energy source has resulted in food webs based on chemolithoautotrophy (Amend & Teske, 2005, Chapelle et al., 2002). Akob and Küsel (2011) listed a wide range of bacteria present at the sub-surface critical zone, all of which are either chemolithoautotrophic primary producers or their heterotrophic consumers, with a distinct lack of photoautotrophic bacteria due to the absence of solar radiation.

1.4. Research aims of the thesis

It is understood that bacteria play a key role in the weathering of geological substrates such as silicates. Despite this, the mechanisms by which this weathering occurs are still largely unstudied. The presence and importance of bacteria at the critical zone have been reported by Chorover et al. (2007); however they concentrated on surface horizons (Figure 1.1; A, E and B), which are rich in biota and organics, whereas the sub-surface critical zone has not yet received much attention despite the rich bacterial community present (Akob & Küsel, 2011). During the development of this research (from 2008), investigations of bacterial weathering at the sub-surface critical zone were in their early stages and had yet to go beyond any rhizospheric influence on microbial communities (Calvaruso et al., 2006, Puente et al., 2004, Uroz et al., 2007). While the role of roots and fungi is important, it is the aim of this thesis to venture into the deeper horizons to deliver the following research goals:

- I. Improve our understanding of the bacterial communities inhabiting the sub-surface critical zone.
- II. Elucidate the role of these bacteria in the weathering process.
- III. In addition, to explore the effect of distinct plant species inhabiting shallow soil horizons on the bacterial communities at depth.

The applications used to detect the presence and decipher the important roles of bacteria at the critical zone are reviewed in the following sections.

1.5. Identification of field sites

In order to address the process of bacterially enhanced weathering in this thesis, it was important to determine a field location that could accommodate sites with distinct vegetation as well as differences in known weathering rates. A field area in western Iceland fulfilled these conditions (Moulton & Berner, 1998, Moulton et al., 2000); previous studies had determined the local weathering potential. Four sampling sites surrounding Skorradalur Lake (TCS1 = 64°30.577N, 21°23.497W; LMS1 = 64°29.279N, 21°31.660W; TCS2 = 64°30.676N, 21°27.191W and LMS2 = 64°29.408N, 21°31.109W) were studied and it was recorded that these sites had different vegetation, yet shared broadly similar basaltic lithology. In addition, the soil profiles at these sites were shallow, permitting ease of access to the rock-soil interface beneath the soil profile.

Vegetation at two of the sampling sites consisted of root-forming higher order plants such as birch and conifer trees (designated TCS hereon in). The distribution of the birch stands were sparse yet evenly distributed with grass patches present across the landscape. Conifer stands were more densely arranged resulting in a lower presence of grass at the soils surface. The remaining sites only supported

lower-order organisms such as lichens and mosses (designated LMS hereon in) and had no evidence of any root forming plants and/or grasses present. Sites supporting higher order plants (TCS1+2) were reported to have a greater rate of weathering than sites supporting only lower order plants (LMS1+2). Moulton and Berner (1998) suggest that the presence of the higher-order plant life enhanced the rate of weathering. The presence of higher-order plants and the associated root systems were believed to influence the microbial diversity, yet whether this differing microbial community had any effect on the efficacy of silicate weathering has yet to be addressed. Hence, these sites in Iceland were selected to examine if the differences in weathering rates between TCS and LMS sites could partially be explained by differences in bacterial communities.

1.6. Introduction to methods

Until the advent of the polymerase-chain reaction (PCR), microbiological studies were largely based on culturable microorganisms. Consequently, the available information on bacteria is skewed heavily towards isolates that are easy to cultivate (Hugenholtz et al., 1998, Rappé & Giovannoni, 2003). However, studies relying solely on culturable organisms ignore the majority of the microbial community that, although not well studied and characterized, may perform vital functions in the biosphere. With the advent of molecular methods of identification, there has been an explosion of the recognised diversity of prokaryote species across the globe (Stotsky, 1997).

Molecular methods are able to determine both culturable and unculturable organisms in environmental samples through the extraction of biomarkers that are unique to individual taxa, in some cases down to species level (Ranjard et al., 2000). These biomarkers can be associated with cell walls, as is the case for

phospholipid fatty acid analysis, whereas many of the newer techniques are based on the ability to extract nucleic acids from cells (Amann et al., 1995).

The use of molecular techniques to identify the presence of bacteria has resulted in the view that bacteria dominate the biosphere, with a diversity and density large enough to play a significant role in the Earth's critical biogeochemical processes (Aslam et al., 2010). Given the high resolution, level of detail and accuracy afforded by modern molecular techniques, the work presented in this thesis uses a number of them to address the above research aims.

1.6.1. Extraction of total nucleic acids from soil

The extraction of total bacterial nucleic acid from soils can be performed using a number of methods. These are broadly grouped into two categories: direct methods and indirect methods. The direct methods are based on lysis of the bacterial cells that occurs while the bacteria are still within the soil matrix; the nucleic acids are separated from both soil contaminants and other cell debris in a single extraction process (Ogram et al., 1987). Indirect methods of nucleic acid extraction are characterized by an initial cell extraction step, in which cells are separated from the soil matrix prior to cell lysis and nucleic acid extraction (Holben et al., 1988). The resulting nucleic acids obtained by these two methods, and derived protocols (Barton et al., 2006, Griffiths et al., 2000, Maarit Niemi et al., 2001, Ogram et al., 1995), show distinct differences in quality and quantity (Esther et al., 2003); direct methods of extraction can result in nucleic acids of a much lower molecular weight and quality than indirect methods but the overall yield may be much greater employing direct methods (Esther et al., 2003). The larger quantities of nucleic acids yielded from a direct extraction are thought to contain a better representation of the total microbial diversity in the soils (Roose-Amsaleg et

al., 2001). However, Stach et al. (2001) suggested that the higher quality and molecular weight of the nucleic acids extracted using indirect methods gave a greater measure of diversity. Esther et al. (2003) supported this claim and concluded that gene banks of a superior nature should be created using indirect methods of nucleic acid extraction from soil samples.

Despite these debates, in this thesis, the decision to use direct or indirect methods of extraction depended on the technical feasibility of the protocols and their appropriateness for the collected samples, as the extraction of nucleic acids from bacteria in andosols is technically challenging (Herrera & Cockell, 2007, Ikeda et al., 2008). The soils collected proved to be more similar in structure to gravel than conventional organic soil. Though defined as a soil due to particle size, the gravel nature of these soils resulted in a low surface area to volume ratio. Moreover, the high metal concentrations within basaltic substrates may also have impeded nucleic acid extraction. Indirect extraction of nucleic acids from the soil matrix resulted in a poor quality and abundance yield. Subsequent analysis was low quality and the results were not repeatable. A higher quality and greater volume of nucleic acids were obtained from these soil samples by employing a direct extraction protocol. The method chosen to be used in subsequent analyses within the thesis was a CTAB and bead beating extraction derived by Griffiths et al. (2000).

1.6.2 Mineral phosphate solubilisation (MPS)

Simple identification of the bacteria present at the sub-surface critical zone does not permit an assessment of the functional role(s) of bacteria in the weathering process. It was therefore necessary to determine the weathering efficacy of sub-samples of the bacteria isolated from the critical zone, at the field sites. The

weathering capability of individual bacterial isolates has been measured previously through the use of mineral phosphate solubilisation (MPS) plates containing an insoluble form of P such as tricalcium phosphate (TCP) or hydroxyapatite (Nguyen et al., 1992, Rodríguez & Fraga, 1999, Rodríguez et al., 2000). The use of MPS plates permits identification of bacterial phenotypes capable of mineral phosphorus solubilisation (MPS phenotypes) through the observation of 'halos' around colonies following incubation; these halos occur through the lowering of the pH surrounding the colony. This is most likely due to the liberation of protons and the release of organic acids during bacterial growth (Goldstein et al., 1999). The insoluble TCP causes the agar on the plates to become opaque and the solubilisation of TCP during bacterial growth clears 'halos' around colonies of MPS phenotypes.

The use of MPS plates offers two sources of information regarding the weathering capability of isolated bacteria: the first is a binary result as to whether a bacterial isolate possesses the MPS phenotype; the second is a continuous measure of weathering efficacy, as the diameter of the halo surrounding the MPS isolates is positively correlated to the weathering efficacy of that isolate under those specific growth conditions (Nguyen et al., 1992). In this study, MPS containing TCP as the insoluble phosphate source was used. The use of TCP simplified the protocol as it was readily available.

1.6.3. Functional gene targeting

The use of MPS plates in the literature has highlighted that a large proportion of bacteria exhibit a MPS phenotype using glucose as the sole carbon source (Jorquera et al., 2008, Kim et al., 1997, Nguyen et al., 1992, Perez et al., 2007, Rodríguez & Fraga, 1999, Rodríguez et al., 2000). Furthermore, bacterial

production of gluconic acid has been discussed as a potential mechanism of biotic weathering (Uroz et al., 2009a).

The production of gluconic acid has been shown to be dependent on the pyrroloquinoline quinone (*PQQ*) and glucose dehydrogenase (*GDH*) genes. The *PQQ* genes have been observed mainly in Gram negative bacteria (Ameyama et al., 1981) and using them would significantly bias results from any environmental samples towards this group of bacteria, whereas previous work has highlighted the importance of the *GDH* gene in gluconic acid production in a wide range of bacterial groups (De Werra et al., 2009, Shigematsu et al., 2005). Hence the successful isolation of the *GDH* gene from *in-vitro* isolated bacteria could represent the first step in the development of an assay to directly extract and quantify this gene from environmental samples. This approach would provide a rapid and inexpensive method for measuring the MPS efficacy from *in-situ* rather than *in-vitro* bacterial community samples.

1.6.4. Soil bacterial community profiles

Identifying every individual bacterium in a soil sample is still not a realistic possibility, the costs and logistics involved restrict the scale of resolution that can be achieved. As with any observational method there is always a balance between the number of organisms identified and the taxonomic level to which these organisms can be identified. One widely accepted method in molecular ecology is the use of community fingerprinting.

Several tools have been adapted to allow researchers to identify greater numbers of microorganisms and/or at higher taxonomic resolution. These include methods such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993) and terminal restriction fragment polymorphism (tRFLP) (Liu et al., 1997). Both of

these methods analyse the bacterial community and provide a snapshot of the community structure. It is also possible to limit the scope and complexity of the community profile by targeting taxonomic groups using taxa-specific designed oligonucleotides (primers), targeting particular domains or individual genera.

These tools provide a quantifiable measure of taxonomic richness and their relative distributions for each individual sample. A general criticism of community profiling is the likely under-representation of the true diversity in the natural environment; with cell numbers as high as 10^9 cells per gram of soil (Aslam et al., 2010) it is evident that the full diversity of prokaryotes cannot be observed and recorded using these methods at present. The limitations in community profiling methods are considered where these techniques are employed in this thesis.

1.6.5. 454 next generation high-throughput sequencing

Over the past three decades, molecular biologists have relied on Sanger sequencing in order to characterize nucleic acid base composition (Sanger et al., 1977). Recently, new methods of short-read, high-volume next generation sequencing (NGS) have been developed, and have resulted in an explosion of research into the rare biosphere (see MacLean et al., 2009 for a review) . Prior to the introduction of NGS, large-scale examinations of the environment were restricted to community fingerprinting methods and small clone libraries (Griffiths et al., 2011). In a bid to obtain a 16S rRNA gene library large enough to be representative of the bacterial community present at the critical zone, NGS was employed throughout the majority of this thesis.

To create the sequence library, 16S rRNA gene bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was carried out by attaching a barcode-labelled tag to a universal bacterial primer and conducting PCR amplification using

the barcode-tagged primer (Acosta-Martinez et al., 2008, Dowd et al., 2008). Subsequent amplicons possess the unique barcode within the initial bases of the 5' end of the nucleic acid. Following amplification and tagging, the amplicons are pooled and processed through a Roche FLX sequencer to obtain nucleotide sequences of the nucleic acid. Following this process each individual sample can be identified and separated from the pool based on the unique barcodes attached to the amplicons prior to sequencing.

The data resulting from 454 NGS requires denoising and quality control checks. The error currently declared is low (0.5%), however the large number of sequences generated by this technique still result in high absolute numbers of sequences expected to contain errors. The denoising step addresses this by clustering sequences together into groups containing similar matching regions and creates a series of consensus sequences. Sequences containing significant error are effectively random and do not conform to any consensus sequence; they are thus 'failed' and removed from dataset. For this study all denoising was conducted using the 'pre.cluster' function available in the Mothur package (Schloss et al., 2009). This is the preferred method of denoising from the Mothur architects and therefore was used here rather than the new 'shhh.seqs' function also available in Mothur. Full details of denoising are discussed by Edgar (2010).

Following denoising, taxonomic data based on the sequences are obtained by employing bioinformatics pipelines. Many commercial establishments, as well as individual researchers each have developed their own bioinformatics pipeline (Caporaso et al., 2010, Schloss et al., 2009). There is not, at present, any consensus on what should be included in each pipeline and as a result it is not possible to make direct comparisons between datasets from different studies. Steps are being taken to create a uniform pipeline but this is currently unavailable.

The use of 454 NGS may already be obsolete, though the data is still viable, as other NGS technologies exist such as Ion Torrent and Illumina. In the case of the Ion Torrent sequencing, the number of sequence reads and sequence lengths are already exceeding those obtained from 454 NGS (Whiteley et al., 2012). Nevertheless, the data obtained during this research are of suitable quality and quantity for use in addressing the stated research aims.

1.7. Specific aims of this thesis

In order to meet the overall objective of measuring bacterial diversity in the sub-surface critical zone, Chapter 2 characterises the bacterial community structure at the sub-surface critical zone at the field site. Rather than relying on culturing and plating techniques, high-throughput technologies were employed to gain a greater understanding of bacterial community composition at the critical zone of sites both with root-forming higher plants (TCS) and without (LMS). A large-scale examination of the bacterial diversity of these sampling areas was conducted using tRFLP analyses to look for obvious differences in the bacterial community fingerprints between soils with different vegetative cover. Details of the taxonomy and diversity were further elucidated using 454 NGS from sub-samples of these sites. The chemical compositions of the soils were measured to determine any relationships between increases in diversity or taxa and specific concentrations of elements and chemical compounds.

Chapter 3 explores how the growth and viability of bacteria are closely linked to the environment the bacterial community inhabit. Environmental variables (for example, temperature, metal concentrations) were measured to gain a better understanding of the conditions to which the bacterial community are exposed at the sub-surface critical zone. Following measurements of the environmental

conditions, *in-vivo* experiments into the weathering capability of isolated bacteria from the critical zone were conducted. The MPS efficacy of each isolate was recorded and measures of bacterial growth rate of isolates were performed under a series of conditions designed to represent the extremes of the environment from which the bacteria were collected.

Chapter 4 investigates the link between diversity, presence, growth and weathering efficacy of bacteria at the critical zone. Microcosm experiments were established to determine which bacteria are responsible for weathering. Chemical analysis of the microcosms' solute release was conducted to measure the occurrence of bacterially-induced weathering as well as indicate possible metabolic by-products of this artificial weathering environment.

Chapter 5 considers the trophic nature of the bacterial community at the critical zone. Given the harsh environment and poor nutrient status of sub-surface critical zone soils, the soil community is likely to have high abundances of heterotrophic bacteria (Akob & Küsel, 2011) and their role in the weathering process is of significant interest (Hunter et al., 1998). However, these environmental conditions do not prohibit the potential for other trophic groups, such as autotrophs, to be present in the environment. Hence, it was important to establish whether heterotrophy is a major function at the critical zone. The presence and dominance of heterotrophy at the critical zone was determined by tracking the flow of carbon from bacterial necromass through the soil community using an isotopically labelled carbon source.

These research chapters, as well as addressing the specific aims of the thesis, intend to provide a general understanding of the sub-surface critical zone bacterial communities, beyond what is currently known.

Chapter II

Bacterial community composition in the critical zone of a basaltic environment: the effects of land-coverage.

The data obtained and presented in this chapter has subsequently been through peer-review and published in the journal: FEMS Microbiology Ecology.

Summers, S., Whiteley, A. S., Kelly, L. C. & Cockell, C. S. Land coverage influences the bacterial community composition in the critical zone of a sub-Arctic basaltic environment. *Fems Microbiology Ecology*, doi:10.1111/1574-6941.12167 (2013).

2.1. Introduction

2.1.1. Why is the sub-surface critical zone important?

Weathering-induced, long term sequestration of atmospheric CO₂ in the form of carbonates is an important Earth-system process (Kump et al., 2000). Discussed in Chapter I was the importance of silicate structures in the weathering process; their ability to weather readily, pooled with the net sequestration of atmospheric CO₂, make these a model substrate for further investigation. The mechanisms of chemical and physical weathering of basaltic structures are well documented (see Dessert et al., 2003 for a review) and the overall rate of weathering quantified (Cochran & Berner, 1996, Dessert et al., 2003, Ellis, 1988, Gislason et al., 1996, Gislason et al., 1996, Navarre-Sitchler & Brantley, 2007). However, previous studies (Ellis, 1988, Gislason et al., 1996, Moulton & Berner, 1998, Navarre-Sitchler & Brantley, 2007) of the solute flux of dissolved ions in rivers and streams are predominately sourced from only chemical and physical weathering, with potential biotic influences amalgamated into a 'black box'. As such, further observations of the environment are required to take biotic processes into account and determine their contribution to the weathering process. The presence of bacteria at the critical zone would suggest they play some role in ecosystem processes, either actively weathering silicates or impacting upon the geochemical processing of the chemical species liberated during weathering.

Root-forming plants play a key role in the weathering of silicate substrates (Moulton & Berner, 1998), particularly mafic materials (Drever, 1994) by breaking down minerals such as biotite, apatite and plagioclase into their constituent elements, which are essential nutrients for the ecosystem. The effect is more pronounced in areas inhabited by fine-root forming plants as the presence of root

structures can increase denudation rates by an order of magnitude compared to similar sites lacking root structures (Cochran & Berner, 1996).

The relationships between plants and soil microbial communities has been investigated previously (see Kent & Triplett, 2002 for a review), for example through processes such as the mycorrhizosphere effect (Barea et al., 2002, Rambelli, 1973) and rhizodeposition (Hartmann et al., 2009, Jones et al., 2004), nutrients are released into the soil matrix that can influence the microbial population. In addition, Wardle et al. (1999) further reported that the bacterial communities from soil inhabited by plants were more active, with activity measured via soil respiration. However the influence of plants on bacterial communities has not addressed the potential for plants to select for bacterial communities which have been implicated in actively weathering silicate structures (Cockell et al., 2009a, Cockell et al., 2009b, Herrera et al., 2008, Thorseth et al., 1995), particularly those which inhabit the sub-surface critical zone.

It is also unclear as to the extent of bacterial weathering and whether this is sufficient to impact on soil nutrient reservoirs. Therefore, the interaction between soils, plants and the bacterial communities at the sub-surface critical zone requires further investigation.

2.1.2. Investigating the bacteria present at the critical zone

Identifying broadly which bacteria are present at the critical zone is necessary prior to identifying the particular taxa responsible for weathering. This chapter describes the investigation into the bacterial communities located at the sub-surface critical zone of the Icelandic field sites using 16S rRNA gene sequence identification and community profiling (detailed in Chapter I). Furthermore, the effect of vegetation

cover on bacterial communities was investigated. Specifically the following hypotheses were tested in this chapter:

- H1. There is a rich diversity of bacteria inhabiting the sub-surface critical zone.
- H2. Bacterial diversity is linked to the chemical composition of the sub-surface critical zone.
- H3. The plant community at the soils surface affects the community structure and diversity of bacteria in the sub-surface critical zone.

2.2. Materials and methods

2.2.1. Sample locations and collection

Soil samples were collected from four sites surrounding Skorradalur lake, Iceland (TCS1 = 64°30.577N, 21°23.497W; LMS1 = 64°29.279N, 21°31.660W; TCS2 = 64°30.676N, 21°27.191W and LMS2 = 64°29.408N, 21°31.109W). The choice of field site was detailed in Chapter I. Sites were located on broadly similar bedrock but differed in vegetative cover. The vegetation at two sites included higher plants (grasses, forbs, and shrubs; TCS1 and TCS2); whereas the other two sites only supported lower plants (mosses and lichens; LMS1 and LMS2). Soil samples were collected from the critical zone at a depth of c. 30 cm (Figure 2.1), below any visible root systems and within the soil horizon C (Figure 1.1; critical zone), which contained rock and mineral fragments that did not exhibit any significant evidence of weathering. Samples were collected aseptically in June 2009 ($n = 18$) and June 2010 ($n = 18$) at sites LMS1 and TCS1; further samples were collected from all four sites in May 2011 ($n = 36$).

Part of this work involved extraction of nucleic acids from soils (section 2.2.2.), however only 50% of samples collected were successfully processed to a quality that was able to provide statistically viable results (Appendix i). This informed the collection of samples in 2011 and a higher number were obtained. This increased sampling effort, while introducing a bias towards the final sampling period, overcame the poor efficiency of nucleic acid extraction from the soils.

All samples were stored in sterile bags (Whirlpak, Fisher Scientific, Loughborough, UK) at ambient outdoor temperature during field work and then frozen at -20°C on return from the field site.



Figure 2.1: Typical excavation conducted to obtain soil samples. Image shown is from lower order plant site (LMS1); the yellow and green scale bar is 30 cm.

2.2.2. Nucleic acid extraction

In order to establish molecular structure and diversity at the sub-surface critical zone it was necessary to obtain a representative yield of molecular biomarkers from all soil samples by extracting nucleic acids. The process of extracting nucleic acids from andosols was problematic for many samples, thought to be due to limited surface area and increased metal contamination (Herrera & Cockell, 2007), therefore optimization trials were conducted (Appendix i and ii).

Ultimately, nucleic acids were extracted from 0.5 g wet weight of soil using the bead-beating phenol extraction method previously described by Griffiths et al. (2000).

2.2.3. Terminal restriction fragment length polymorphism (tRFLP) analysis.

Bacterial communities were characterised by terminal restriction fragment length polymorphism (tRFLP) analysis, a low-cost, high-throughput method for measuring molecular diversity that allows the differences between microbial communities

from each site to be visualised and these differences to be statistically analysed. Bacterial communities from 72 samples taken between June 2009 – May 2011 from all sites were analysed.

The 16S rRNA gene was partially amplified using the primers 63f (6FAM) and 530r. The amplification was carried out using 1 µL of sample template DNA in a 50 µL reaction containing 250 nM of each primer, 2 mM MgCl₂, 0.1 mM of each of the deoxynucleoside triphosphates, 5 µg bovine serum albumin (BSA; New England Biolabs, MA, USA) and 1.75 U of Taq with 5 µL of 10X buffer (New England Biolabs, MA, USA). The conditions of the PCR reactions consisted of an initial denaturation for 90 seconds at 94°C; followed by 35 cycles of: 45 seconds at 94°C; annealing for 60 seconds at 55°C and elongation for 3 minutes at 72°C. A final elongation step was also performed at 72°C for 10 minutes. PCR products were verified on a 1.25% w/v agarose gel by electrophoresis.

PCR products were purified using an Illustra™ GFX™ PCR clean up kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's guidelines. Purified PCR products were quantified using a Nanodrop spectrophotometer (NanoDrop technologies, Montchanin, DE, USA) according to the manufacturer's instructions. PCR product (50 ng) was digested in a 10 µL reaction containing 10 U *MspI* restriction endonuclease and 1 µL of enzyme buffer (New England Biolabs, MA, USA), along with 1 µg BSA. Digestion was carried out for 4 hours at 37°C. Digested product (2 µL) was added to 9 µL of Hi-Di formamide and 0.35 µL of Liz 600 abiotic size standard (Applied Biosystems, CA, USA) was used to confirm the length of each fragment measured. Capillary gel electrophoresis was then performed on a 3730 DNA sequencer (Applied Biosystems, CA, USA) to obtain electropherograms for each sample, presenting the abundance and fragment

length for each amplicon measured. Analysis of electropherograms was done using Genemarker software (SoftGenetics, PA, USA), and fragment peaks corresponding to individual tRFs were binned manually. Terminal restriction fragments (tRFs) shorter than 50 nucleotides (n.t.) in length or with an intensity lower than 50 units were not included. Relative abundances were then calculated for each tRF within a sample.

Only 40 samples yielded sufficient quality and quantity to be included in tRFLP analysis (Table 2.1). This was due to poor nucleic acid extraction efficacy of the protocol on andosol samples. Subsequent analyses therefore included 26 samples from LMS sites and 14 from TCS sites.

Table 2.1: A list of the samples that yielded suitable PCR products for tRFLP analysis. Given are the sample location and a description of the dominant land coverage for that site. Samples indicated by “*” were selected for 454 next-generation sequencing (Section 2.2.4).

Sample Number	Sample site	Land Coverage
1	LMS	Lichen and Mosses
2	LMS	Lichen and Mosses
3	LMS	Lichen and Mosses
4	LMS	Lichen and Mosses
5*	LMS	Lichen and Mosses
6	LMS	Lichen and Mosses
7	LMS	Lichen and Mosses
8	LMS	Lichen and Mosses
9	LMS	Lichen and Mosses
10*	LMS	Lichen and Mosses
11	LMS	Lichen and Mosses
12	LMS	Lichen and Mosses
13	LMS	Lichen and Mosses
14	LMS	Lichen and Mosses
15	LMS	Lichen and Mosses
16	LMS	Lichen and Mosses
17	LMS	Lichen and Mosses
18	LMS	Lichen and Mosses
19	LMS	Lichen and Mosses
20	LMS	Lichen and Mosses
21	LMS	Lichen and Mosses
22	LMS	Lichen and Mosses
23*	LMS	Lichen and Mosses
24	LMS	Lichen and Mosses
25	LMS	Lichen and Mosses
26	LMS	Lichen and Mosses
27	TCS	Predominately Birch Stands
28	TCS	Predominately Birch Stands
29	TCS	Predominately Birch Stands
30	TCS	Predominately Birch Stands
31	TCS	Predominately Birch Stands
32*	TCS	Predominately Birch Stands
33*	TCS	Predominately Birch Stands
34	TCS	Predominately Birch Stands
35*	TCS	Predominately Conifer Stands
36	TCS	Predominately Conifer Stands
37	TCS	Predominately Conifer Stands
38	TCS	Predominately Grass and Birch
39	TCS	Predominately Grass and Birch
40	TCS	Predominately Grass and Birch

2.2.4. 454 next-generation sequencing (NGS)

To allow taxonomic identification of tRFs and provide a phylogenetic examination of the bacterial communities it was necessary to obtain 16S rRNA gene sequences for a portion of the samples examined in this study. Samples were selected for sequencing based on the presence of unique tRF peaks observed in the tRFLP analysis (section 2.2.3). This provided the greatest probability of obtaining accurate identifications for individual tRFs.

Bacterial communities in 3 TCS and 3 LMS samples collected in June 2010, were sequenced using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) directly using a Roche 454 FLX Genome sequencer at the Research and Testing Laboratory (Lubbock, Texas, USA). Briefly, the bTEFAP was carried out using a barcode tag attached to a universal bacterial primer (63f) and a non-barcode primer (530r) during amplification of 16S rRNA gene, prior to sequencing. Subsequent removal of the barcode tag and primers, as well as analysis of all sequences was conducted using MOTHUR software (Schloss et al., 2009) to generate rarefaction curves and filter sequences. The number of sequences compared for each sample in the rarefaction analysis was limited to c. 2500 as this was a level of sub-sampling permitted all samples to be analysed without including any bias to the results. Chimeric sequences were removed with the ChimeraSlayer package (Haas et al., 2011) using QIIME software (Caporaso et al., 2010). Phylogenetic trees were generated using the FastTree (v2) package in QIIME (Price et al., 2009) by converting all sequences to representative operational taxonomic units (OTUs; 97% sequence similarity). All sequences were aligned against the Silva sequence database, as this is a regularly updated and curated database (Pruessle et al., 2007) and taxonomy allocated using the Naïve Bayesian rRNA Classifier v1.0 analysis tool within RDP classifier (Wang et al.,

2007b). Phylogenetic trees were visualized using the Interactive Tree of Life (ITOL) software (Letunic & Bork, 2011).

2.2.5. *In-silico* digestion of 454 NG sequences

Solitary tRF peaks may distinguish between taxonomic groups but do not provide taxonomic identification. To address this, individual tRFs were assigned an approximate taxonomic identity through *in silico* digestion of 454 NG sequences (see section 2.2.5). All sequences were analysed using TRFLPMAP software (http://nebc.nerc.ac.uk/cgi-bin/trflp0_2.cgi) using the restriction endonuclease *MspI* cleavage site. As some tRFs can consist of several different taxa, the taxa of all sequences with the same cleavage site were identified and the approximate taxon assigned to each tRF was equal to the taxon represented by >75% of all sequences.

2.2.6. Cell count enumerations

To determine the total cellular biomass at the critical zone, total cell numbers from 5 LMS and 5 TCS soils were extrapolated. Five replicates were selected as this gave enough replication to infer biomass for the remainder of samples from each site. Cell number was determined by adding 0.55 g (wet weight) of soil to 5 mL sdH₂O. Added to the slurry was 500 µL of the nucleic acid binding dye SYBR[®] Green I DNA (0.1% w/v stock; Invitrogen, Paisley, UK) was added to the sample and incubated at 20°C in a dark environment for 1 hour.

To assist detachment of cells from the soil matrix, the solution was briefly sonicated using a sonicated water bath (Scherrer AG, Wil, Switzerland) for 10 seconds at 20°C at a low frequency setting, to prevent cellular lysis. To capture cells, 100 µL of solution was passed through a 0.2 µm black polycarbonate filter and then washed with 100 µL of sdH₂O.

Cells were enumerated using a epifluorescent Leica DMRP microscope (Leica Microsystems, Bensheim, Germany) with an excitation wavelength of 450 – 490 nm and a long band cut off filter of >515 nm. All enumerations were conducted by counting 50 fields of view per sample to obtain a mean count per sample and then correcting for dilution to give values of cells per gram soil.

2.2.7. Chemical analysis

Soil chemical analyses were performed to investigate relationships between bacterial diversity and soil chemical properties. The major elements present at the critical zone at sites LMS1 and TCS1, collected in 2009, were determined by X-Ray Fluorescence (X-RF) spectrometry. Samples were prepared by fusing one part powdered soil sample with five parts FluXana flux (20% lithium tetraborate w/w, 80% lithium metaborate w/w) at 1000°C. Samples were then poured into a mould and cooled to room temperature, forming a glass disc. These glass discs were analysed using an ARL 8420+ dual goniometer wavelength-dispersive XRF spectrometer. The analysis was conducted as previously described (Ramsey et al., 1995, Watson, 1996).

2.2.8. Soil organic carbon

The concentration of soil organic carbon (SOC) was measured in samples collected from sites LMS1 and TCS1 using loss on ignition (LOI; $n = 18$). Samples were oven dried at 100°C overnight to obtain a dry weight, and SOC-free weight was obtained following 1 hour's combustion at 500°C as described by Heiri et al. (2001). SOC concentrations were calculated by subtracting SOC-free weight from oven-dry weight.

2.2.9. Soil pH

Given that pH is known to be a major factor determining bacterial diversity (Fierer & Jackson, 2006), it was important to determine the pH of the soils being examined. Measurements of soil pH were conducted on 18 samples from sites LMS1 and TCS1. These soils were made into a 1:2.5 slurry (soil:H₂O) according to Suzuki et al. (2009). pH was measured using a Hydrus 300 digital pH meter (Fisher Scientific, Loughborough, UK) equipped with a HI-1230B electrode (Hanna Instruments, UK).

2.2.10. Statistical analysis

All statistical analyses were carried out using the Primer (v5) package (Clarke & Warwick, 2001) or R v2.15.1 (R Development Core Team, 2010) using the vegan package (Oksanen et al., 2012). A visual evaluation of the chemical composition of samples at each site was conducted using Canonical Correspondence Analysis (CCA) ordination. The ENVFIT function in Vegan was used to observe individual variables related to dissimilarity in ordinations. Confirmation of observed dissimilarities were tested using analysis of similarity (ANOSIM).

Statistical differences in OTU richness from 454 NGS was determined using a Mann Whitney U test of difference. A rarefaction curve was generated to visualize OTU richness of 16S rRNA genes from all six samples.

Visualization of tRF dissimilarity was conducted using Principles Component Analysis (PCA), using the Bray-Curtis measure of dissimilarity. Measures of tRF diversity were obtained by calculating the Shannon-Wiener (Shannon-Weaver) diversity index (H'), although attributing diversity indices for community fingerprints provides an inherent error (Blackwood et al., 2007). Nevertheless, this method was appropriate for comparisons between sample sites but comparisons of these results to other studies may be inaccurate. To determine which tRFs had the

greatest influence on differences between bacterial communities, a SIMPER analysis (Clarke & Warwick, 2001) was carried out.

One-way analysis of variance (ANOVA) was used to test for differences between sample sites for pH and SOC. Non-parametric data, that did not meet the assumptions required for parametric analysis, was analysed using the Mann-Whitney U test; these included cell enumerations and differences in diversity indices H' .

2.3. Results

2.3.1. Terminal restriction fragment length polymorphism (tRFLP) analysis.

The bacterial communities from both TCS and LMS sites were examined using community fingerprinting (Figure 2.2 a&b). This provided a measure of the relative abundances of individual terminal restriction fragments (tRFs).

Bacterial communities from both sample sites showed clusters in PCA ordination (Figure 2.3), with overlap in the groupings indicating similarity between communities. However the bacterial communities differed significantly between sites (ANOSIM, $R = 0.224$, $p = 0.005$). SIMPER analysis of tRF relative abundances revealed that 37.65% of the observed dissimilarity between sample sites could be attributed to 16% of the tRFs (Table 2.2). Through comparisons of tRF abundance and *in-silico* fragments of the 454 sequences, taxonomic affiliations for each tRF were established. These tRFs represented *Proteobacteria* (*Alpha*, *Beta* and *Gamma* classes) and *Acidobacteria* predominately. *Gemmatimonadetes* and *Bacilli* accounted for 1.95% and 1.45% of community differences observed, respectively. Only 3.4% of the observed dissimilarity was accounted for by other taxa.

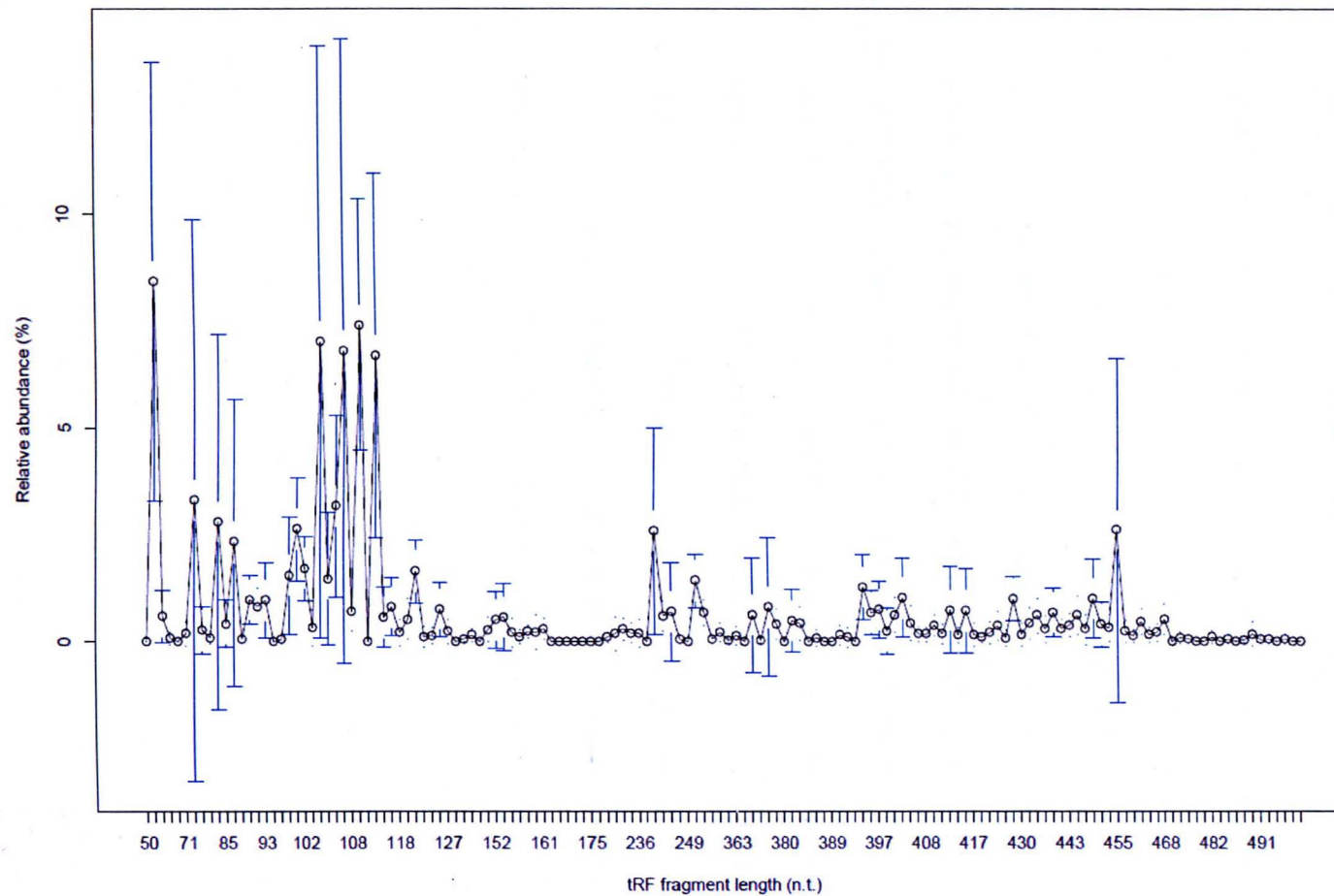


Figure 2.2a: Frequency plots showing the tRF relative abundance and lengths for LMS site. Error bars = 95% CI

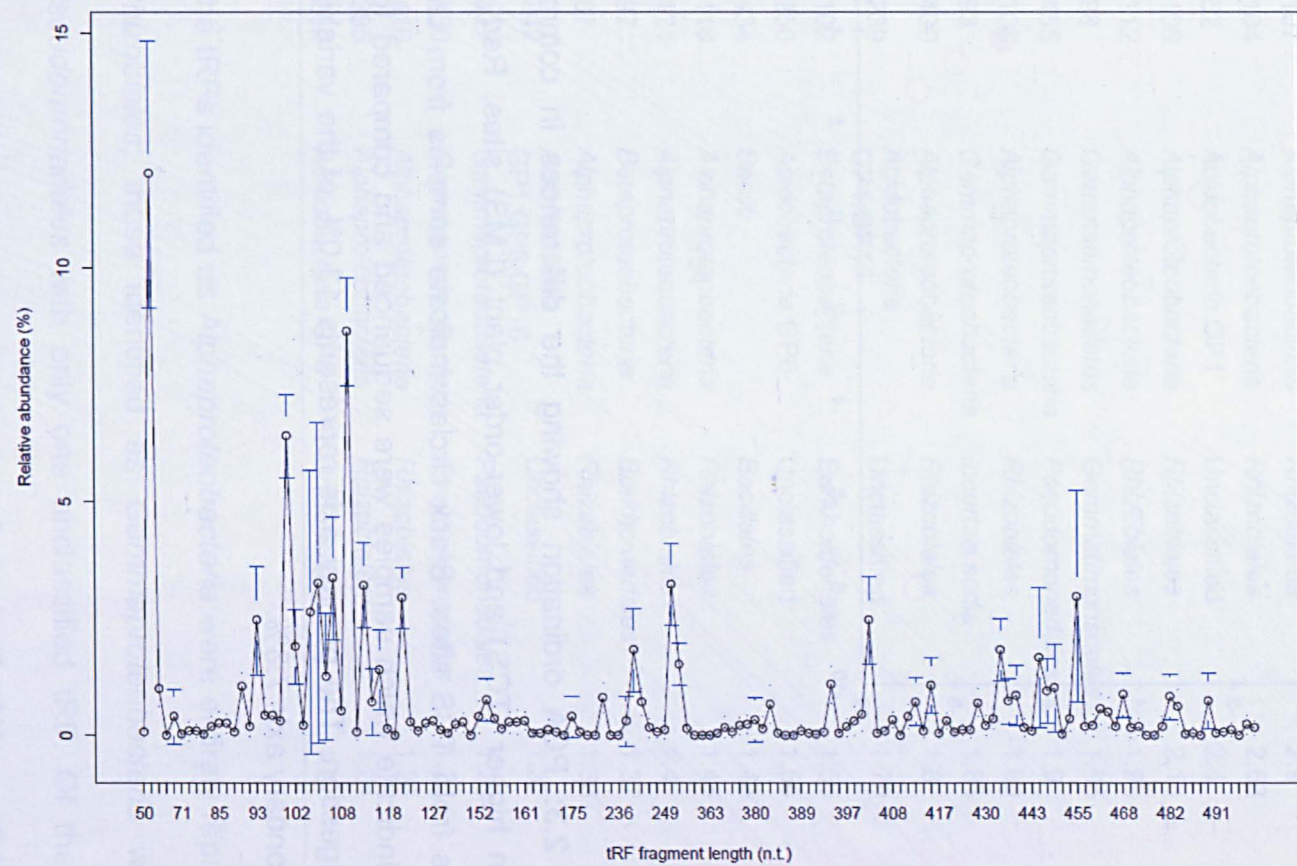


Figure 2.2b: Frequency plots showing the tRF relative abundance and lengths for TCS site. Error bars = 95% CI

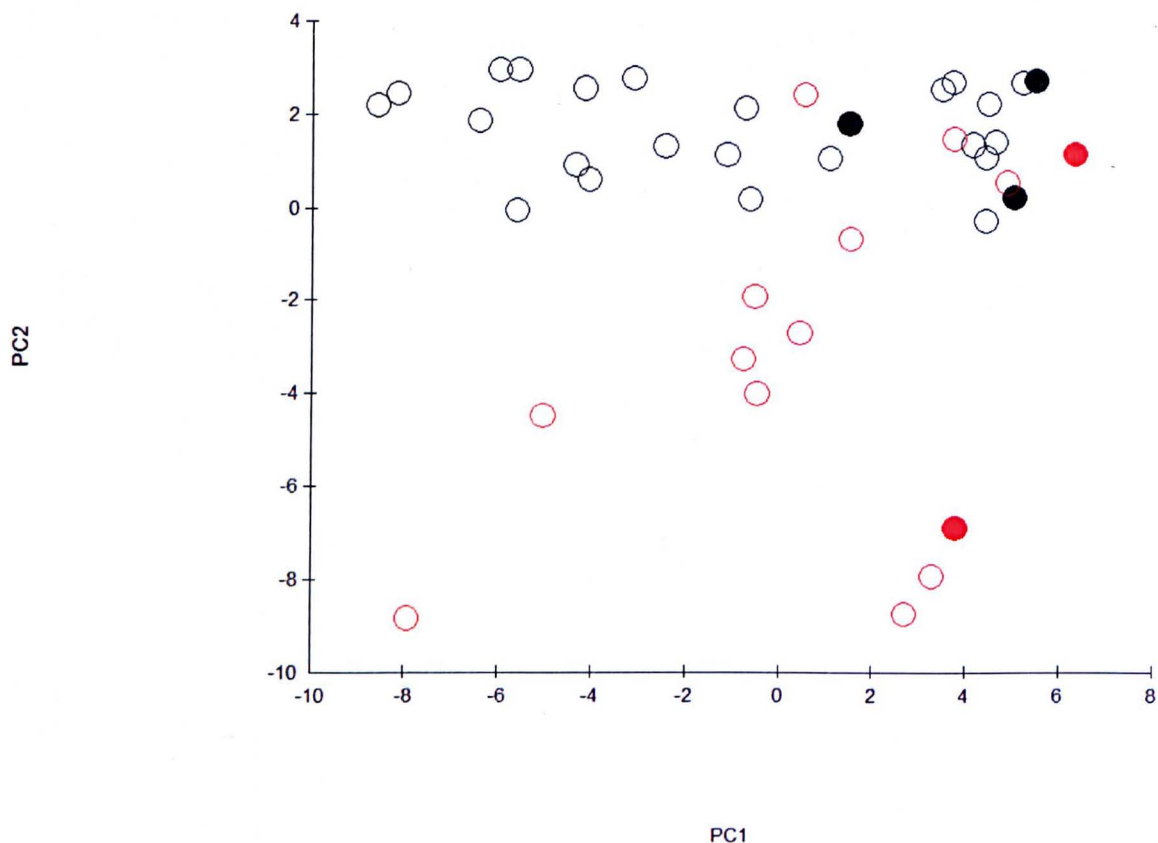


Figure 2.3: PCA ordination showing the differences in community structure between higher (TCS) and lower-order plant (LMS) sites. Red circles indicate samples from TCS sites. Black circles indicate samples from LMS sites. Filled circles indicate which samples were sequenced and compared to tRFs using *in silico* digestion. The primary axis represents 13.0% of the variability in data and the secondary axis 7.3%.

Table 2.2: Results of SIMPER analysis showing the cumulative effect of individual tRFs on bacterial community dissimilarity between higher- (TCS) and lower-order plant (LMS) sites. Only taxa with an individual contribution > 1.1% are shown.

tRF length (n.t.)	Taxon (Class)	Taxon (Order)	Individual contribution (%)	Cumulative contribution (%)
107	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	2.9	2.9
104	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	2.63	5.53
52	<i>Acidobacteria</i> GP1	Unclassified	2.4	7.93
106	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	2.18	10.11
112	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	1.98	12.09
98	<i>Gemmatimonadetes</i>	<i>Gemmatimonadales</i>	1.95	14.04
455	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	1.9	15.94
105	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	1.89	17.83
93	<i>Gammaproteobacteria</i>	<i>Incertae sedis</i>	1.85	19.68
400	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	1.8	21.48
239	<i>Acidobacteria</i> GP1:GP18	Unclassified	1.79	23.27
102	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	1.59	24.86
250	<i>Acidobacteria</i> GP6	Unclassified	1.56	26.42
434	<i>Bacilli</i>	<i>Bacillales</i>	1.45	27.87
116	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	1.45	29.32
121	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	1.4	30.72
97	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	1.37	32.1
87	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	1.36	33.46
242	<i>Acidobacteria</i> GP1:GP6:GP18	Unclassified	1.32	34.78
447	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	1.29	36.07
450	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	1.28	37.35
416	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	1.26	38.62
395	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	1.24	39.85
252	<i>Acidobacteria</i> GP6	Unclassified	1.2	41.05

The tRFs identified as *Alphaproteobacteria* were entirely represented by the order *Rhizobiales*; those identified as *Gammaproteobacteria* were represented by *Pseudomonadales* with only one unclassified tRF. Of the *Proteobacteria*, the *Betaproteobacteria* had the least influence (2.96%) on the difference between sample sites; with all representatives from the order *Burkholderiales*. Individual tRFs identified as *Acidobacteria* had representatives from three groups GP1, GP6 and GP18, of which GP1 had the largest influence (2.4%) on dissimilarity between

sites. The remaining 60% of differences between bacterial communities of each sample site were accounted for by 127 individual tRFs, each tRF contributing <1.1% of observed difference (data not shown). The α -diversity of tRFs, given as Shannon diversity indices (H'), was significantly greater at LMS sites (H' ; 3.14 ± 0.41 , $n = 26$) than at the TCS sites (H' ; 2.86 ± 0.33 , $n = 14$) (Mann Whitney U test, $U = 607$, $p < 0.04$).

2.3.2. 454 next-generation sequencing (NGS)

The partial 16S rRNA gene sequences obtained using 454 NGS from six samples revealed the contribution of the abundant classes of the bacterial communities to each sample site (Figure 2.4). The data from the 454 NGS resulted in a total of 15,315 16S rRNA gene sequences with a mean read length of >350 bases.

Bacterial communities at the critical zone were dominated by seven classes of Bacteria in both sites, with c. 90% of all Bacteria identified as *Alpha*-, *Beta*-, *Delta*- and *Gammaproteobacteria*, *Actinobacteria*, *Gemmatimonadetes* and *Acidobacteria*. Across both sites, *Alphaproteobacteria* were the most abundant (c. 34%) followed by *Acidobacteria* GP6 (c. 17%), *Gammaproteobacteria* (c. 11%), *Acidobacteria* GP1 (c. 6%), *Deltaproteobacteria* (c. 6%), *Betaproteobacteria* (c. 5%), *Gemmatimonadetes* (c. 4%) and *Actinobacteria* (c. 3.5). However the relative abundances of these bacterial phylogenetic groups differed between TCS and LMS sites.

There was no observable difference in relative abundance for three common taxa (*Deltaproteobacteria*, *Betaproteobacteria* and *Actinobacteria*). However, there were observed differences in the relative abundance of other taxa. *Alphaproteobacteria* made up 38% of sequences at the TCS sites yet only 28% of sequences at the LMS sites. This was also observed for *Gammaproteobacteria*

though variability was high at TCS sites offering less confidence. Conversely, *Acidobacteria* and *Gemmatimonadetes* were less abundant in the TCS samples.

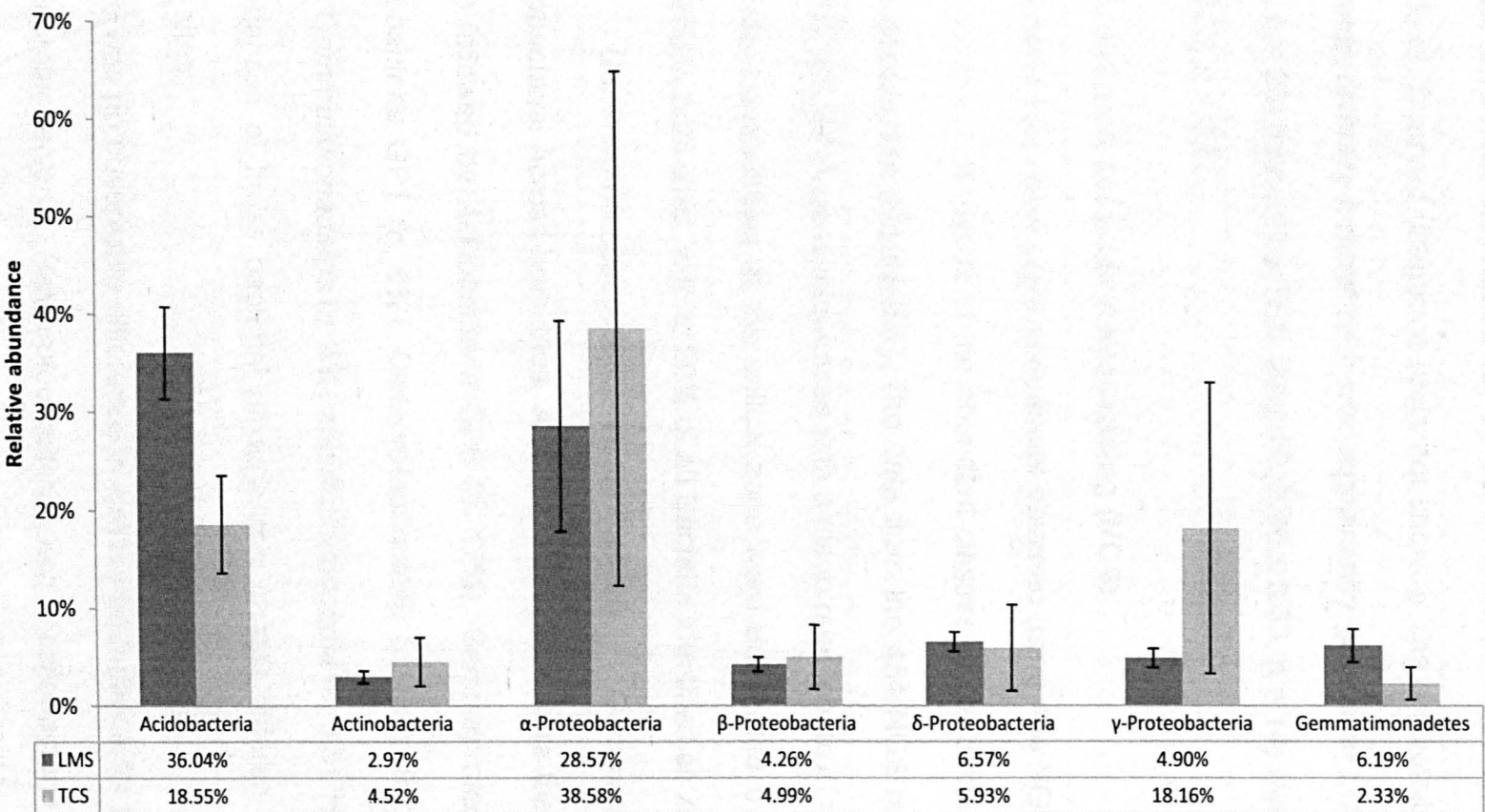


Figure 2.4: Differences in relative abundance for the seven most common taxa identified from the critical zone; the LMS site is indicated by dark grey shading and TCS site by light grey shading. Error bars indicate standard errors for $n = 3$. The values given for each taxa are the mean relative abundance of 16S rRNA sequences.

The overall qualitative taxonomic units (OTUs; 97% sequence similarity) richness from the soil samples appears to be high as rarefaction curves

did not reach asymptote, even after 2500 sequences were examined for each sample (Figure 2.5). Comparisons of 16S rRNA gene sequences rarefaction curves showed that the α -diversity was lower at TCS sites. The general bacterial composition of critical zone soil communities was compared by examining differences in the numbers of OTUs. OTU richness at each sample site was higher at LMS (621.5 ± 45.7) than at the TCS (504.5 ± 51.8 ; Mann–Whitney U-test, $U = 36$, $p = 0.004$). However, this assessment of bacterial diversity did not take account of OTU evenness or abundance. In total there were 172 OTUs that were common to both sample sites (Table 2.3).

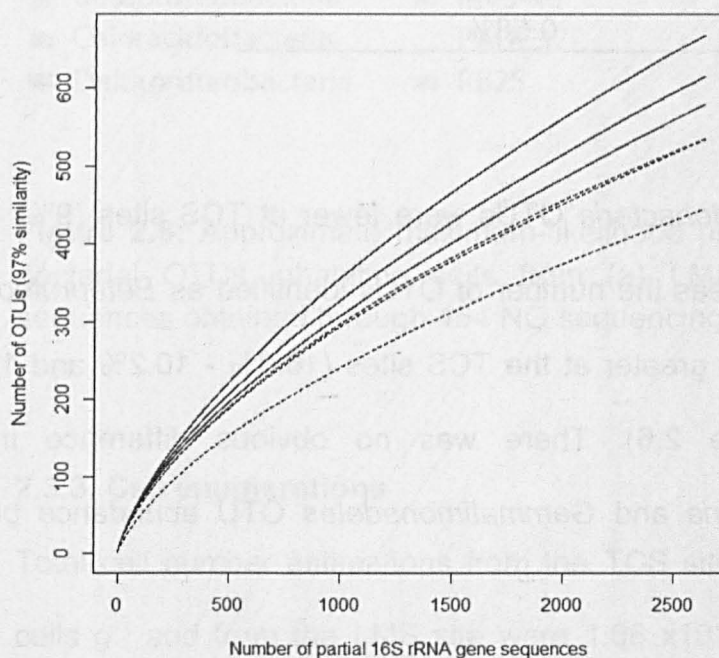


Figure 2.5: Rarefaction analyses, indicating the α -diversity of bacterial communities at TCS and LMS sites. Solid lines indicate LMS sites and broken lines indicate TCS sites.

Table 2.3: Taxonomic identity of OTUs common to both sample sites (TCS and LMS). Individual OTUs grouped into taxonomic Order.

Order	Relative abundance
<i>Rhizobiales</i>	25.58%
<i>Acidobacteria-Incertae sedis</i>	16.86%
<i>Acidobacteriales</i>	12.79%
<i>Pseudomonadales</i>	9.88%
<i>Sphingomonadales</i>	8.14%
<i>Burkholderiales</i>	5.81%
<i>Oceanospirillales</i>	5.23%
<i>Xanthomonadales</i>	3.49%
<i>Rhodospirillales</i>	2.91%
<i>Chromatiales</i>	2.91%
<i>Solibacterales</i>	2.33%
<i>Caulobacterales</i>	1.16%
<i>Actinomycetales</i>	0.58%
<i>Procabacteriales</i>	0.58%
<i>Myxococcales</i>	0.58%
<i>Holophagales</i>	0.58%
32-20	0.58%

The number of *Acidobacteria* OTUs were fewer at TCS sites (9%) than at LMS sites (14.5%), whereas the number of OTUs identified as *Betaproteobacteria* and *Actinobacteria* were greater at the TCS sites (16.8% - 10.2% and 11.1% - 7.0% respectively; Figure 2.6). There was no obvious difference in *Alpha-* or *Gammaproteobacteria* and *Gemmatimonadetes* OTU abundance between LMS and TCS sites.

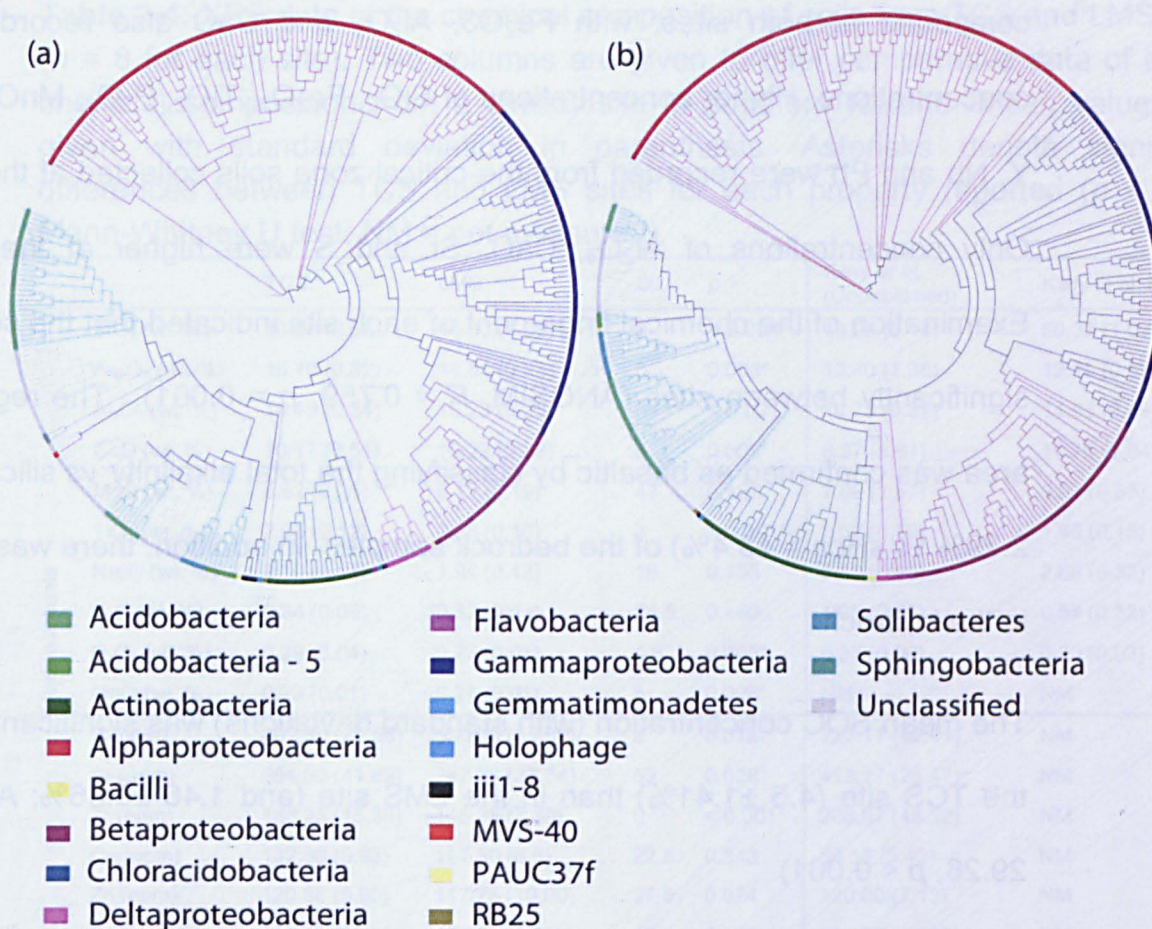


Figure 2.6: Approximate maximum-likelihood phylogenetic tree of representative bacterial OTUs inhabiting soils from (a) LMS sites and (b) TCS sites. All sequences obtained through 454 NG sequencing.

2.3.3. Cell enumerations

Total cell number estimations from the TCS site were 2.25×10^7 ($\pm 2.03 \times 10^7$) cells g^{-1} and from the LMS site were 1.06×10^7 ($\pm 0.96 \times 10^7$) cells g^{-1} . The cell numbers were significantly greater at the TCS site (Mann-Whitney U test, $U = 54924.5$, $p < 0.001$).

2.3.4. Soils chemical properties

The chemical properties of the critical zone soils from LMS and TCS sites were measured using XRF elemental analysis (Table 2.4). SiO_2 was the most abundant

compound at both sites, with Fe_2O_3 , Al_2O_3 and CaO also recorded in high concentrations. Higher concentrations of SiO_2 , Fe_2O_3 , TiO_2 , P_2O_5 , MnO , V , Zr , Sc , Y , Nb and Pb were recorded from the critical zone soils collected at the TCS site. Only concentrations of Al_2O_3 , CaO , Sr and S were higher at the LMS site. Examination of the chemical fingerprint of each site indicated that the soils differed significantly between sites (ANOSIM, $R = 0.759$, $p = 0.001$). The regolith of the area was confirmed as basaltic by classifying the total alkalinity vs silica (salinity = 2.79% vs silica = 48.4%) of the bedrock samples. In addition, there was c. 3% PO_3 in the bedrock.

The mean SOC concentration (with standard deviations) was significantly higher in the TCS site ($4.5 \pm 1.41\%$) than in the LMS site (and $1.46 \pm 0.86\%$; ANOVA, $F = 29.26$, $p < 0.001$).

Mean soil pH (with standard deviations) was significantly lower in the TCS (6.56 ± 0.11) compared to the LMS (6.94 ± 0.08) sites (ANOVA, $F = 62.48$, $p < 0.001$).

Table 2.4: XRF data of the chemical composition of soils from TCS and LMS sites ($n = 8$ for each site). Two columns are given to offer comparative data of basalt chemical composition from a similar site in southern Iceland. Mean values are given with standard deviation in parenthesis. Asterisks denote significant differences between TCS and LMS sites for each property reported ($p < 0.05$; Mann-Whitney U test; NM = not measured).

	TCS	LMS	U	p	Kelly <i>et al.</i> (Unpublished)	Kelly et al. (2011)	
Major elements	SiO ₂ (wt. %)	46.95 (0.86)	44.53 (1.08)	0	< 0.001*	49.29 (3.44)	50.32 (1.07)
	Fe ₂ O ₃ (wt. %)	15.70 (0.32)	14.81 (0.53)	5	0.003*	12.40 (1.38)	12.15 (0.59)
	Al ₂ O ₃ (wt. %)	14.15 (0.34)	15.23 (0.25)	64	< 0.001*	14.73 (0.37)	14.53 (0.49)
	CaO (wt. %)	10.17 (0.51)	10.22 (0.38)	5	0.003*	8.37 (1.81)	10.75 (0.84)
	MgO (wt. %)	5.92 (0.26)	6.12 (0.19)	47	0.127	7.08 (1.87)	6.07 (0.63)
	TiO ₂ (wt. %)	2.95 (0.12)	2.68 (0.10)	2	< 0.001*	2.52 (0.56)	1.68 (0.15)
	Na ₂ O (wt. %)	2.10 (0.17)	1.94 (0.12)	16	0.103	3.48 (0.85)	2.66 (0.32)
	K ₂ O (wt. %)	0.34 (0.06)	0.32 (0.03)	24.5	0.460	1.05 (0.55)	0.64 (0.32)
	P ₂ O ₅ (wt. %)	0.29 (0.04)	0.23 (0.01)	4.5	0.005*	0.37 (0.06)	0.20 (0.03)
	MnO (wt. %)	0.23 (0.01)	0.21 (0.01)	5	0.006*	NM	NM
Trace elements	V (ppm)	407.50 (20.56)	380.00 (15.80)	9	0.018*	227.17 (42.41)	NM
	Sr (ppm)	294.50 (41.59)	347.38 (37.74)	53	0.028*	413.17 (25.47)	NM
	Zr (ppm)	188.25 (15.39)	145.38 (7.39)	0	< 0.001*	268.67 (43.32)	NM
	Cu (ppm)	132.38 (9.93)	127.50 (6.5)	22.5	0.343	58.17 (5.42)	NM
	Zn (ppm)	120.88 (5.00)	117.75 (10.00)	27.5	0.674	120.00 (7.13)	NM
	Cr (ppm)	110.00 (25.13)	129.88 (8.31)	47	0.128	229.67 (17.99)	NM
	Ba (ppm)	105.50 (8.14)	114.88 (12.38)	46.5	0.141	217.83 (49.02)	NM
	Ni (ppm)	61.50 (9.53)	68.38 (5.78)	47	0.128	124.67 (26.88)	NM
	Co (ppm)	49.38 (1.41)	50.88 (2.17)	48	0.099	42.00 (5.44)	NM
	S (ppm)	44.50 (27.32)	244.25 (133.53)	63	< 0.001*	187.83 (56.57)	NM
	Sc (ppm)	41.38 (2.77)	38.38 (1.06)	8	0.012*	22.67 (3.08)	NM
	Y (ppm)	35.45 (4.82)	27.80 (1.01)	0	< 0.001*	38.52 (5.46)	NM
	Nb (ppm)	22.06 (1.73)	17.43 (1.15)	1	< 0.001*	31.53 (5.29)	NM
	Ga (ppm)	21.63 (1.06)	21.00 (0.76)	21	0.244	22.50 (0.84)	NM
	Rb (ppm)	7.00 (2.39)	4.38 (1.19)	11	0.28	18.17 (4.07)	NM
	Pb (ppm)	3.00 (1.2)	2.00 (1.41)	19	0.014*	2.83 (1.17)	NM
	Th (ppm)	2.38 (0.74)	1.25 (1.16)	14	0.054	3.17 (1.47)	NM
	U (ppm)	1.38 (0.52)	0.52 (1.04)	28.5	0.732	1.33 (0.82)	NM
	As (ppm)	0.88 (1.36)	1.36 (1.07)	24.5	0.394	1.50 (1.38)	NM

The relative proportions of the major elements from this study are similar to those obtained from other studies. The proportions of Na, Fe and K exhibit differences in proportion.

Comparisons of XRF chemical fingerprints with tRF relative abundances indicated that *Acidobacteria* (tRF 52 n.t.), *Gemmatimonadetes* (tRF 98 n.t.) and *Alphaproteobacteria* (tRFs 112 and 110 n.t.) were significantly correlated with the

overall chemical composition of the LMS site, while the TCS site chemical composition showed a correlation with tRF peak 85 n.t. which was most closely aligned with *Nitrospira* or *Acidobacteria* taxa (Figure 2.7).

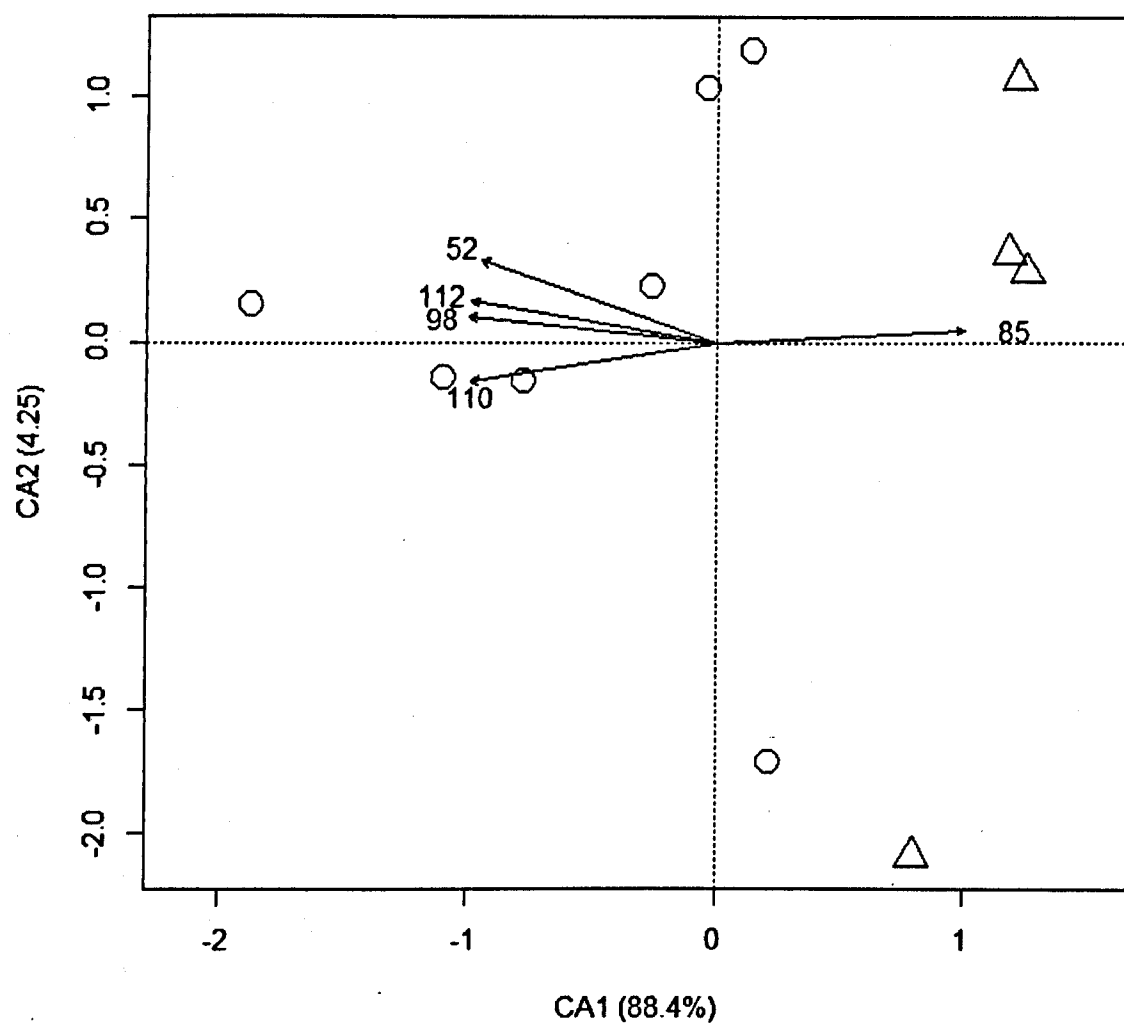


Figure 2.7: CCA bi-plot showing the elemental composition dissimilarity of soils harbouring higher- and lower plants with tRF lengths. LMS sites are indicated by circles and TCS sites indicated by triangles. Statistically significant correlations between samples and relative abundances of tRFs are presented as vectors ($p \leq 0.05$).

2.4. Discussion

This chapter characterises the soil and bacterial communities in the sub-surface critical zone of two Icelandic field sites that differ in vegetation and weathering rates (Moulton & Berner, 1998, Moulton et al., 2000).

2.4.1. Plant influences to bacterial diversity

The presence of higher plants, the resulting higher concentrations of SOC generated through leaf litter decomposition, and the expected associated root exudates and tannins, are likely to influence soil bacterial communities, resulting in the greater abundance of *Proteobacteria* measured here. There are numerous sub-taxa within the *Proteobacteria* capable of enhancing mineral weathering (Uroz et al., 2007). Additionally, higher plants can alter the chemical composition of surrounding soils (Calvaruso et al., 2009); including pH (Augusto et al., 2000), which potentially affects *Acidobacteria* abundance.

In order to maximize mineral leaching from the soil matrix, some plants, through the provision of root and mycorrhizal exudates, have been shown to actively encourage populations of mineral weathering microbes (Calvaruso et al., 2006, Uroz et al., 2007, Uroz et al., 2011a). Differing species of higher order plants are known to influence the mineral weathering efficacy of bacterial communities from forest soils (Collignon et al., 2011). In this study the two different higher-plants (Beech and Conifer spp) would be expected to influence the sub-surface bacterial communities differently due to differences in root structure, root exudates, leaf litter quantity and litter composition. However, further investigation of the influence of individual tree species on sub-surface critical zone soil bacterial communities in

cold environments would be required to confirm these effects and relate them to the differences observed in the bacterial communities studied in this chapter.

2.4.2. Bacteria inhabiting the sub-surface critical zone

Similar to studies of surface soils (see Janssen, 2006 for a review), bacterial communities identified in this thesis, as inhabiting the sub-surface critical zone, were dominated by *Acidobacteria* and *Proteobacteria*. *Proteobacteria* have previously been shown to be abundant in Arctic soils (Neufeld & Mohn, 2005), with undisturbed tundra being dominated by this group. *Alphaproteobacteria* have specifically been reported as a dominant member of Arctic soils (Neufeld & Mohn, 2005, Zhou et al., 1997). Bacterial communities at the critical zone of LMS and TCS sites studied here were found to be dominated by *Alphaproteobacteria*, *Acidobacteria* and *Gammaproteobacteria* and are important components of soil bacterial diversity.

In temperate soils the ratio between *Alphaproteobacteria* and *Acidobacteria* has been proposed to be an indicator of soil nutrient status and bacterial diversity (Eilers, 2011, Fierer et al., 2007, Griffiths et al., 2011). Furthermore, the ratio between *Alphaproteobacteria* and *Acidobacteria* has been shown to be linked to the type of vegetation present at the soil surface (Griffiths et al., 2011, Thomson et al., 2010). The ratio of *Alphaproteobacteria* to *Acidobacteria* in previous studies has been calculated as 2.25 and 1.44 for vegetated and bare soils, respectively (Thomson et al., 2010). For this study, based upon relative abundance of tRFs matching *Alphaproteobacteria* and *Acidobacteria*, resulting ratios of 3.09 and 1.74 were recorded here for TCS and LMS site, respectively. Therefore, the hypothesis raised here is that the ratios of *Alphaproteobacteria* to *Acidobacteria* are based upon nutrient status between the sites and reflect the differential nutrient inputs

from TCS and LMS sites. For example, *Acidobacteria* are thought to be oligotrophic (Fierer et al., 2007), whereas *Proteobacteria* encompass a large copiotrophic contingent (Simonato et al., 2010). The lower SOC observed in the LMS sites may permit *Acidobacteria* to outcompete *Alphaproteobacteria*, as the formers carbon requirements are best suited to the lower concentrations of SOC in the LMS site. This hypothesis may explain the community structure differences observed at the study sites and further expands the presumption that overarching ecological rules govern the presence of key microbial taxa, such as observed in other studies (Fierer et al., 2007) and may also extend to cold Arctic environments.

2.4.3. Bacterial community similarity between sample sites

Although the bacterial communities at each sample site are significantly different, the 16S rRNA gene sequences indicated that c. 50% of OTUs recorded at either site were common between sites. Of these common OTUs, >99% were represented by the phyla *Acidobacteria* and *Proteobacteria*. These similarities between bacterial communities at each sampling site were visualized by PCA (Figure 2.3). This showed bacterial communities from the LMS site varied more compared to communities from the TCS site. The overlap of communities demonstrates that in a few particular samples, bacterial communities were more similar between sites than within sites. One explanation for this may be spatial limitations on the radius of influence that plant species have on the soil microbial communities, resulting in community similarity decreasing with distance from the plants. Spatial limits to the influence of plant rhizodeposition can be between 20 – 40 cm (Miniaci et al., 2007). For example, the LMS sites were more bacterially diverse; this may reflect communities at TCS sites being a more specialized core of taxa that are capable of processing of available nutrients input to the

environment by plants. As these samples were collected at depths of c. 30 cm from areas without visible evidence of root systems, they may be at the limits of this rhizospheric influence. It is possible to address this spatial influence on bacterial communities by using transects to examine the diversity of bacteria at set distances from plant stands; however, this was not undertaken in this study.

A higher abundance of *Actinobacteria* in samples obtained from the TCS sites was apparent from the 16S rRNA gene sequences, yet this was not corroborated by tRF SIMPER examination. Furthermore, the number of *Actinobacteria* OTUs identified by NGS was lower in samples from TCS sites, indicating greater abundance yet lower diversity of this taxon. *Actinobacteria* have been found in terrestrial lithic environments studied for mineral weathering (Abdulla, 2009, Cockell et al., 2009a, Cockell et al., 2009b, Kelly et al., 2010) and are believed to enhance weathering through the production of siderophores and filamentous growth (Cockell et al., 2009a). A greater proportion of *Actinobacteria* at TCS sites would be expected given the higher SOC concentrations, as *Actinobacteria* are known to be important organisms in carbon cycling (Goodfellow & Williams, 1983). The lack of clear differences in *Actinobacteria* between sampling sites in this study may be explained by Hamdali et al. (2012). They showed that siderophores alone may not permit growth in P-limited oligotrophic environments, such as the LMS sites here, which would potentially limit *Actinobacteria* growth at the LMS sites. They reported that *Actinobacteria* isolates required the production of organic acids in addition to siderophores to actively enhance mineral weathering. However cultured isolates obtained in the present study from the LMS sites included *Actinobacteria* species capable of mineral phosphate weathering (Chapter III). An

attempt to confirm organic acid production was attempted in chapter III, however this proved unsuccessful.

2.4.4. Bacterial use of organic carbon

Carbon is an important element in soil ecosystems and can act as a significant driver in bacterial ecology (Nielsen et al., 2011). The higher numbers of cells observed in the TCS site compared to the LMS site (section 2.3.3.) may be linked to higher concentrations of SOC (section 2.3.4.) that likely result from the increase in biotic matter from the plants present, both from leaf litter decomposition and rhizospheric deposition. Higher concentrations of SOC may provide a significant energy source for the predominately heterotrophic bacterial community thought to inhabit the sub-surface (Hunter et al., 1998). This implies a mutually beneficial environment stimulating plant and bacterial growth (Radutoiu et al., 2003). Moreover, the increased abundance of *Rhizobiales* associated with the higher order plants in the TCS sites may be responsible for N₂ fixation (Deslippe et al., 2006). This potential for diazotrophic behaviour combined with the TCS sites higher concentrations of SOC and root exudates may potentially enhance bacterial cell numbers at the cost of other taxa such as Archaea (Karlsson et al., 2012). Moreover, whilst the higher SOC measured at the TCS site does coincide with higher cellular biomass, the LMS site contained lower SOC concentration and cellular biomass. However, the enumeration methods used in this study were not able to differentiate between bacteria and archaea.

2.4.5. The ecological influence of pH on bacterial communities

Alteration of soil pH is evident in this study as the TCS site had a lower soil pH than the LMS site (section 2.3.4.). However, the *Acidobacteria* population was also

higher at the LMS site, which was unexpected given the pH of the soils. Previous work in the Arctic and in temperate soils has shown that pH influences microbial diversity, with low pH values coinciding with high *Acidobacteria* numbers (Chu et al., 2010, Griffiths et al., 2011). However (Fujimura et al., 2012) showed that *Proteobacteria* dominated some low pH volcanic soils from the island of Miyake, Japan while *Acidobacteria* were more abundant in soils with a higher pH of c. 6 in Beech (*Fagus spp.*) stands; suggesting that plants may confound the influence of pH on the ratio of these two taxa.

This study corroborates the findings of Fujimura et al. (2012), as the TCS site had significantly lower pH than the LMS site, yet harboured a larger proportion of *Proteobacteria*, specifically *Alphaproteobacteria* in this case. Although pH is a major factor in determining microbial diversity, the small yet significant differences in pH recorded in this study, may have been overridden by the presence of plants and associated nutrient deposition. The influence of vegetation coverage on bacterial diversity was also observed by (Griffiths et al., 2011); however, the effects of the vegetation were superseded by the highly significant effects of pH on bacterial diversity.

2.4.6. Linking bacterial abundance to weathering

In this study it is not possible to directly link the elemental abundances observed in the soils to bacterial weathering. Many elements co-vary with several environmental and chemical factors. For example, phosphorus (P) is bound by aluminium (Al) species (Kimble et al., 2000, Lukito et al., 1998). The lower concentration of P in the LMS site might be caused by the higher concentrations of Al, rather than a lower rate of P liberation from rock apatites by chemical or biological processes. However, other elements are more likely to reflect

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weathering rates. The use of Si as an indicator of weathering basaltic rock has been described previously (Navarre-Sitchler & Brantley, 2007) and the higher concentration of Si measured for the TCS site suggests either that the lower pH associated with the site causes a higher release of Si into the soil and/or the biota is causing an enhanced release of Si from the rocks through similar hydrolytic processes. Other elements such as Na and Ti, that are also elevated in the TCS sites, are likely to reflect rock weathering.

As the bedrock at each site shared broadly similar lithology any differences in soil compositions may be due to the differing biology present at each site (Moulton & Berner, 1998, Moulton et al., 2000). Correlations between soil XRF elemental abundance of the samples and the respective tRF peaks suggest a relationship between bacterial abundance and the broad chemistry of soils (Figure 2.7). The dominant tRF peaks corresponded to *Proteobacteria* and *Actinobacteria* and showed significant correlations with spatial ordinations of soil chemical properties; however, further analysis of the data was not able to show any significant relationship between individual bacterial taxa and a chemical species.

2.5. Conclusions

In conclusion, this chapter contains a study of the bacterial community present at the sub-surface critical zone of a cold basaltic environment in two sites with different plant covers. The critical zone was dominated by *Proteobacteria* and *Acidobacteria*. Despite significant differences in chemical composition between the two sites (H2), which may be correlated to the different rates of weathering, amongst other factors, by the presence of plants and differences in pH, this study shows that the soil critical zone at both sites shared 50% of bacterial OTUs (97% sequence similarities). These data suggest that a core of closely-related taxa may

inhabit the nutrient-poor environment of the cold volcanic critical zone (H1), but that overall composition is likely influenced by other factors such as pH, organic carbon availability and the rate of weathering itself. For example, the LMS sites were more bacterially diverse (H3), and may reflect communities at TCS sites being more specialized in the processing of available nutrients afforded to them by plants through competitive exclusion processes. Future studies must determine whether the organisms that inhabit the critical zone are a) optimally adapted to this environment. The sub-Arctic environment may have a significant effect on both the weathering capability and the diversity of the bacterial communities, Furthermore how these communities may alter during the extreme seasonal variation of these latitudes; b) whether the bacterial communities are capable of contributing actively to weathering of the silicate substrate and c) whether they are just passive inhabitants of the environment or could be classed as ecological engineers.

Environmental controls of bacterial growth and phosphate weathering capability

3.1. Introduction

Microbial communities are known to modify their environments through growth and phenotypic expression of physiologies such as mineral phosphate solubilisation (MPS; Kim et al., 1997, Richardson, 2001, Rodríguez & Fraga, 1999). In turn, growth rates and phenotypic traits such as MPS are closely controlled/limited by a range of environmental factors, such as temperature (O'Brien & Lindow, 1989) as this is a major determinant of bacterial growth (Antoniou et al., 1990, Novick, 1955, Ratkowsky et al., 1982). However, Štyriaková et al. (2012) presented evidence that while temperature does appear to influence biological weathering, the mechanisms are not simplistic. They show that the order of favourable ion release from basaltic substrate is altered depending on the temperature of the environment. Both Fe and Si were shown to be retained within substrates to a greater extent in cold weathering experiments (c. 4°C). Therefore, the temperature of the sites studied in this thesis may influence bacterially mediated processes within the critical zone such as weathering (Calvaruso et al., 2006, Uroz et al., 2009b, Uroz et al., 2011a), although the specific ions liberated may depend on the environmental temperatures.

Nautiyal et al. (2000) discussed the importance of acid production, amongst other processes, in the ability of bacteria to weather insoluble phosphate minerals. Determining if organic acids, such as gluconic acid, are produced by bacteria through glucose metabolism could therefore represent an important step in confirming the presence of this phosphate solubilisation phenotype in the environment. Several studies to date have presented evidence for rock weathering and phosphate release through the process of gluconic acid production by bacterial isolates known to weather mineral phosphate (Goldstein et al., 1993,

Welch & Ullman, 1999, Wu et al., 2008). Therefore, by identifying bacterial community production of organic acids associated with weathering, simple functional gene assays of the metabolic cycles involved in organic acid production (Figure 3.1), such as the glucose dehydrogenase (*GDH*) gene, could be performed on environmental samples as an *in-situ* proxy for weathering capability.

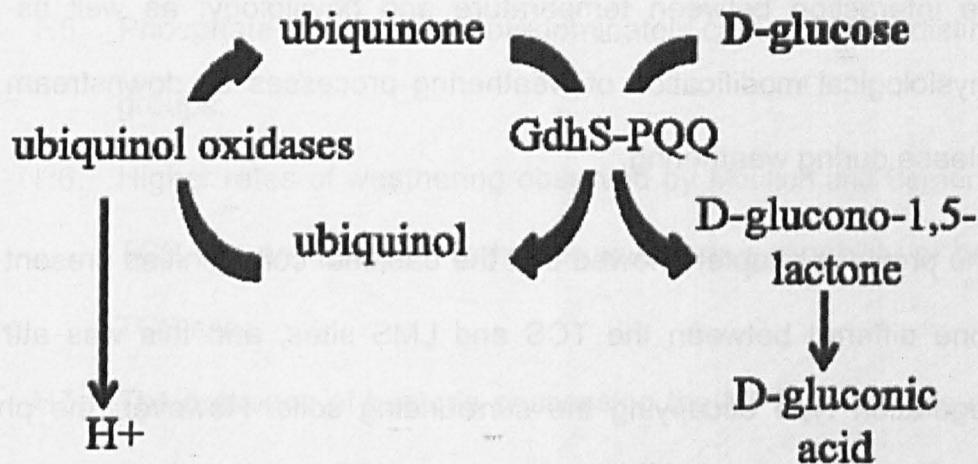


Figure 3.1: A simplified schematic illustrating a potential pathway for glucose metabolism to create gluconic acid using the enzyme *GDH*. This reaction results in the production of protons and organic acid, both of which are key processes involved in bacterial weathering. Figure obtained from Štyriaková et al. (2012).

Other key factors which are hypothesised to be rate limiting to bacterial weathering concern the materials liberated into the ecosystem during the weathering process. Moulton and Berner (1998) suggested that measured differences in weathering rates among sites identified at Skorradalur Lake would also lead to differences in the bio-availability of metals such as Mn, Fe, Zn and Mn. These four metals all play a key role in microbial processes in the soil. For example, Cu is required for certain enzymes (e.g. oxygenases and cytochrome-c oxidase), Zn is a micronutrient, Fe can be a catalyst for N₂ fixation and Mn is required for the production of superoxide dismutase. However, all of these trace metals are known to cause deleterious effects to growth above certain concentrations (Rajapaksha et al.,

2004). Subsequently, increased availabilities of these metals could secondarily impact upon weathering rates through, for example the effect of liberated heavy metals on bacterial growth (see Giller et al., 2009 for a review, Leita et al., 1995). Previous work (Chapter II) indicated different microbial diversity and metal concentrations at the different study sites, and thus, a key factor to be resolved is the interaction between temperature and physiology; as well as the potential physiological modification of weathering processes by downstream heavy metal release during weathering.

The previous chapter showed that the bacterial communities present at the critical zone differed between the TCS and LMS sites, and this was attributed to the vegetation type occupying the surrounding soils. However, the physiology and potential weathering activity of these bacterial communities was not addressed and represent a knowledge gap in understanding the causes for the differential diversity observed between sites. Although vegetation plays a key role in structuring bacterial diversity (Nusslein & Tiedje, 1999, Thomson et al., 2010, Wardle et al., 1999), in these specific ecosystems, secondary regulation may also be occurring due to the action of temperature regime, weathering rates themselves and/or the liberation of metal components. However, the results of biotic and abiotic factors influencing weathering usually co-vary and, as a result, disentangling them *in-situ* is problematic. Thus, an *in-vitro* approach was applied here.

The physiological limitations to bacterial growth and the efficacy with which the bacterial community was able to liberate phosphate from minerals into the local environment was investigated and the underlying mechanism was assessed.

3.1.1. Aims

The research presented in this chapter aimed to investigate the environmental conditions of the sub-surface critical zone and determine if these conditions were physiologically limiting bacterial growth and secondary weathering processes by addressing the following hypotheses:

- H4. Bacteria at the critical zone inhabit an optimal region for bacterial growth.
- H5. Phosphate weathering is predominately carried out by distinct phylogenetic groups.
- H6. Higher rates of weathering observed by Moulton and Berner (1998) from the TCS site are reflected in higher weathering capability of bacteria from the TCS site.
- H7. The presence of bacteria possessing the MPS phenotype can be detected *in-situ* using a *GDH* gene assay.

3.2. Materials and methods

The general methods used here have been described in detail in previous chapters. Where appropriate, the reference to the relevant section is given and only additional or new methods are given here in detail.

3.2.1. Sampling sites

Two sampling sites were selected for this study (TCS1 and LMS1), details of which are given in Chapter 2. For a full description of sample sites refer to Moulton et al. (2000).

3.2.2. Sample collection

Soil sampling occurred in June 2010. Nine 500 g soil samples were collected from the critical zone, c. 30 cm below soil surface. All samples were collected aseptically and stored in sterile plastic bags (Whirlpak, Fisher Scientific, Loughborough, UK) at ambient outdoor temperature (c. 11°C) then at 4°C upon return to the laboratory. Aliquots of 10 g were taken from each sample and pooled together to be used as subsequent inoculants. Samples were stored no longer than 4 weeks before experimental incubations were established.

3.2.3. Soil temperature

To monitor temperature at the critical zone, U12-008 dataloggers (Tempcon instrumentation, Arundel, UK) linked to TMC6 HB external thermistors were left *in-situ* at the TCS and LMS sites for 23 months from June 2009 to May 2011. Each data logger was set and positioned to record hourly readings for the temperature at the critical zone and air temperature immediately above the sample site.

In addition to temperature, solar radiation at the surface of site TCS1 was measured to assess links between photosynthetically active radiation (PAR) and sub-surface critical zone temperature. This was measured every ten minutes during a ten month period using a PAR probe (Onset, Borne, Mass, USA) connected to a Hobo Micro Station Data Logger (Onset, Borne, Mass, USA). No PAR data was collected for site LMS1 due to a technical failure caused by local biological disturbances.

3.2.4. The concentration of bioavailable metals at the critical zone

Although the data presented previously (Chapter II) illustrated the concentrations of metals in the critical zone, there was no indication that these were biologically accessible forms. In this chapter, diethylenetriaminepentaacetic acid (DTPA) extractions were conducted to extract the soluble metal ions from the soils as described by Liang and Karamanos (1993), as these soluble ions would also be bio-available.

Following extraction of soluble metal ions, all solutions were stored in 5% HNO₃ acid solution at -80°C. Each sample was analysed in triplicate for concentrations of bioavailable Cu, Zn, Fe and Mn. DTPA extracted solutions were diluted (10^{-1}) with 5% HNO₃ solution and ICP-AES (Leemna, Prodigy spec, NH, USA) analysis was carried out as per Yang et al. (2008) using the following standards and wavelengths: Cu (324.754 nm), Fe (239.563 nm), Zn (213.856 nm), Mn (259.372 nm).

3.2.5. Bacterial isolations

In order to isolate organisms present at the critical zone, low-organic solidified growth media was prepared. Tryptic soy agar (BD Difco, Oxford, UK) was diluted

to a 10^{-1} concentration and supplemented with agar (Oxoid, Basingstoke, UK; Bacterial Agar No1) to a final concentration of 1.5% containing 0.5 g of sterile crushed soil. Crushed soil was autoclaved at 121°C for 30 minutes before being added to provide a source of any organics specific to the critical zone.

Soil bacteria were isolated from soil slurries generated by adding 1 g (wet weight) of soil sample to sdH_2O in a 1:1 mixture. Plates were inoculated with 30 μL of soil solution and incubated for up to 120 hours. In order to increase the diversity of culturable bacteria, a range of aerobic conditions and temperatures were applied during the incubations (Table 3.1). A proportion of plates were incubated at 20°C (Model 305, cooled incubator; LMS LTD, Kent, UK), whilst all other plates were incubated at 10°C. This lower temperature was expected to promote psychrotolerant and psychrophilic species (see Morita, 1975 for definitions) that may inhabit the Icelandic sub-surface critical zone soil samples. Isolates grown anaerobically were cultured using sealed chambers with anaerobic environment generation pouches (AN0035, Oxoid, Hampshire, UK).

Individual colonies were subcultured to obtain pure isolates, which were then incubated in aerobic conditions at 10°C (Model 305, cooled incubator; LMS LTD, Kent, UK); this ensured that all organisms were obligate or facultative aerobes. This method resulted in >100 microbial isolates. As bacteria were the focus of this study, all visually discernible non-bacterial isolates (e.g. fungi etc.) were discarded, leaving 79 culturable candidates for further downstream physiological experiments.

For long term storage of isolates, all organisms were aseptically transferred to a 50% lysogeny broth (LB) with 50% glycerol solution in sdH_2O (1:1 vol/vol) and stored at -80°C.

Table 3.1: Initial growth conditions used to isolate various bacterial strains from critical zone soil samples.

Incubation temperature (°C)	Aerobic/anaerobic environment	Soil inocula	Treatment
10	Aerobic	LMS soil	A
20	Aerobic	LMS soil	B
20	Anaerobic	LMS soil	C
10	Aerobic	TCS soil	D
20	Aerobic	TCS soil	E
20	Anaerobic	TCS soil	F

3.2.6. Determination of isolate taxonomy

Taxonomic affiliation of bacterial isolates was achieved through sequencing of the 16S rRNA gene (hypervariable regions V1-V9). Domain Bacteria universal primers 27f and 1541r were used in a colony PCR to amplify the 16S rRNA gene of cultured isolates. Amplification was performed in a 50 µL reaction containing 300 nM of each primer; 5 µL of 10X Taq buffer; 2 mM MgCl₂; 0.2 mM of each of the deoxynucleoside triphosphates; and 2.5 U of Taq (New England Biolabs, MA, USA).

PCR conditions consisted of: initial denaturation for 10 minutes at 94°C to promote cell lysis; followed by 30 cycles of 1 minute at 94°C; annealing for 40 seconds at 58°C; elongation for 40 seconds at 72°C; with a final elongation step for 10 minutes at 72°C. All PCR products were verified on a 1% (w/v) agarose gel by gel electrophoresis and correct size PCR products stored at -20°C.

Six isolates failed to amplify using primers 27f and 1541r and were amplified using universal primers 63f and 530r to yield partial 16S rRNA sequences (V1-V3) using the amplification conditions described in Chapter 2. All PCR products were purified using an Illustra™ GFX™ PCR clean-up kit (GE Healthcare, Buckinghamshire, UK) prior to Sanger termination sequencing.

For all PCR products, Sanger sequencing was conducted using the Big Dye terminator and X-terminator kits (Applied Biosystems, CA, USA) with a 3730 DNA sequencer (Applied Biosystems, CA, USA) as per the manufacturer's guidelines.

Subsequent phylogenetic sequence analysis of all sequences was conducted using MOTHUR software (Schloss et al., 2009) to filter sequences based on quality prior to alignment against the Silva sequence database (Pruesse et al., 2007). Phylogenetic tree generation was conducted in MEGA (Tamura et al., 2011) using Jukes-Cantor (Jukes et al., 1969) nucleotide substitution model and 1,000 bootstrap replicates. Visualization of trees was performed using Interactive Tree of Life (ITOL) software (Letunic & Bork, 2011).

Taxonomic classification of sequences was performed using the Naïve Bayesian rRNA Classifier v1.0 analysis tool within the Ribosomal Database Project II website (Michigan State University) (<http://rdp.cme.msu.edu/>). Classifications with a confidence threshold of 95% were based on the taxonomical hierarchy proposed in Bergey's Manual of Systematic Bacteriology, release 6.0 (Garrity et al., 2004).

3.2.7. Growth measurements of isolates under stress

To simulate the conditions experienced by the bacteria *in-situ*, and to measure the effect of these conditions on bacterial growth, microcosms were established simulating the chemical and thermal properties measured at the sub-surface

critical zone. Only Cu concentrations differed significantly between samples sites (Section 3.3.2.), therefore, this element was selected for use in growth trials.

Randomly selected isolates were subcultured aerobically on fresh low-organic media (as described above) at 20°C for 48 hours. Colonies were resuspended in 20 μL sdH_2O then added to 350 μL of 10% lysogeny broth (LB). Colony inocula were exposed to two experimental variables: Cu and temperature. For temperature, the inocula ($n = 35$) were grown at a range of temperatures between 1 and 22°C (1, 5, 10, 15 and 22°C \pm 0.1°C) using a Bioscreen C reader (Labsystems Oy, Helsinki, Finland). For Cu, the inocula ($n = 15$) were grown at 22°C (\pm 0.1°C) at Cu concentrations of increasing order of magnitude from 0.1 mM to 100 mM (0.1, 1, 10 and 100 mM) using a Bioscreen C reader (Labsystems Oy, Helsinki, Finland). To achieve experimental Cu concentrations CuSO_4 was supplemented into growth media until desired molarity was achieved.

Optical density (OD) absorbance readings were measured at 600 nm every 15 minutes during incubation, following 15 seconds of medium oscillation shaking of the microplate. Incubation and OD measurements continued until all isolates had reached stationary growth phase. Growth rates (μ) were calculated according to Equation 3.1, where N_0 is the initial OD at time t_0 and N_x is the OD at time t_x . The base-10 logarithmic phase of growth curves were converted to natural logarithms (\ln) by multiplying by 2.303. Time was given in hours; therefore μ was expressed as h^{-1} . As t_0 and t_x were required to be within the exponential growth phase for accurate calculation, these variables were individually determined for each isolate. Consequently, the final formulae were unique to each isolate's growth curve. This analysis was performed in duplicate for each isolate and the means reported.

$$(3.1) \quad \mu = \frac{2.303(\log N_x - \log N_o)}{(t_x - t_o)}$$

3.2.8. Mineral phosphate solubilisation

To determine if any of the isolated bacteria possessed a mineral phosphate solubilisation (MPS) phenotype, MPS plates were prepared as described by Mailloux et al. (2009) using tri-calcium phosphate (2.5% w/vol, TCP; VWR international, Lutterworth, UK) as a the solid-state mineral phosphate source and glucose as the sole carbon source (1% w/vol). Bacterial isolates were inoculated onto the MPS plate following standardisation to equal optical densities (OD = 0.5). The insoluble TCP causes the agar to become opaque; solubilisation of TCP by bacteria with the MPS phenotype clears the agar, forming halos around the growing colonies during incubation (Figure 3.2). Thus, MPS plates permitted the identification of MPS phenotypes associated with isolated bacteria through simple observation of halos around bacterial colonies following incubation.

Isolated colonies were cultured using MPS media at 20°C for 60 hours. Halos surrounding bacterial colonies were identified and digital images taken. The area of individual halos was determined using ImageJ software package (v1.45, National Institutes of Health, USA) with radii of plates used as a datum (90 mm). Positive controls using a weak HCl acid (5%) and *Pseudomonas aeruginosa* were included to confirm chemical and biological media solubilisation, respectively. *P. aeruginosa* was selected as the positive control as it has been shown to produce gluconic acid and possesses a MPS phenotype (Bagyaraj et al., 2000).

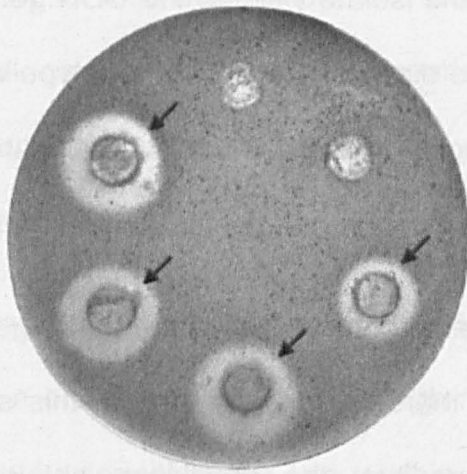


Figure 3.2: An example of the halos that are present surrounding microbial isolates exhibiting the MPS phenotype. Isolates marked with arrows represent the MPS phenotype. Those isolates without arrows show no halo and do not possess the ability to solubilise mineral state phosphate. Image shown has been modified from the original (Nico et al., 2012).

3.2.9. Glucose dehydrogenase gene isolation

Under the assumption that gluconic acid was being produced by MPS isolates and that this physiological trait could be regulated by the presence of the *GDH* gene, a functional assay was conducted to attempt and detect the *GDH* gene from the bacterial isolates in this study. The aim of this approach was to attempt and correlate the presence of the *GDH* gene with the possession of the MPS phenotype. This would yield a simple PCR that could act as a proxy for the presence or absence of the bacterial MPS phenotype *in-situ*. Currently the most common methods for detecting organic acids responsible for weathering involve laboratory examination of VFAs from cultured organisms, or total sample organic acid assays, such as high performance liquid chromatography (HPLC; Andersson & Hedlund, 1983, Jones, 1998, Wang et al., 2007c), neither of which differentiate between kingdoms within a complex soil environment.

To amplify the *GDH* gene, a primer set was chosen that was designed based on sequence comparisons of several isolates from *Proteobacteria* taxa (Shigematsu et al., 2005). All bacterial isolates ($n = 56$) were amplified using the primers *GDH-f1* and *GDH-r1* according to the protocol of Shigematsu et al. (2005). In order to optimise the protocol for isolates analysed here and confirm sequence specificity

of the primers, isolate AW1 8, the isolate yielding the *GDH* gene fragment closest to the published length and also displaying a MPS phenotype was amplified using 12 annealing temperatures ranging from 50.1°C to 65.3°C with product specificity and size confirmed by 1% (w/v) agarose gel electrophoresis.

The positive and negative bacterial controls described by Shigematsu et al. (2005) were financially inhibitive and therefore not included in this study. The simplest alternative approach to ensure that the amplicons obtained were accurate amplifications of the *GDH* gene was to amplify, measure fragment length and sequence the amplicons to ensure that these matched published versions of the *GDH* gene.

Finally, products originating from the PCR optimization using isolate AW1 8, were gel extracted using an Illustra™ GFX™ PCR clean-up kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer's guidelines and the nucleotide sequence determined using Sanger sequencing (as described in this chapter) to confirm target gene specificity. The initial intent was to amplify this single isolates *GDH* gene and following confirmation that the assay was accurate, roll this out to the remaining isolates used throughout the temperature and MPS assays.

3.2.10. Statistical analysis

The concentration of bioavailable metals at the critical zone was examined statistically. The means of triplicate samples were calculated and statistical comparisons conducted utilizing the Mann-Whitney U test as the data did not meet the assumptions for a parametric analysis.

To determine whether individual factors such as initial cultivation temperature, aerobic state or sample site resulted in differences in MPS efficacy, a Mann-

Whitney U test was conducted. Due to the multiple factors nested within the overall growth treatments, comparisons of each treatment and the resulting differences in MPS efficacy were determined using a Kruskal-Wallis test of difference.

Differences in bacterial growth at various environmental temperatures were tested using a one-way ANOVA. The high number of isolates selected for this stage of the study permitted the use of this parametric test to determine differences in growth rate between the temperatures employed. Normality of data was confirmed using the Anderson Darling test prior to ANOVA.

Comparisons between growth rates at the lowest experimental Cu concentration and optimal growth rates from temperature trials were unbalanced. This required a Mann-Whitney U test of differences in growth rates under the two growth conditions. Comparisons between Cu concentrations and their effect on bacterial growth were conducted using a one-way ANOVA, following Anderson Darling test for normality.

3.3. Results

3.3.1. Temperature through the soil profile

Recorded air and sub-surface critical zone temperatures from LMS1 and TCS1 sites, spanning a 23 month period, displayed a clear pattern of seasonal variation at both sites and both depths (Figure 3.3). Air temperatures in the summer months reached a high of 32.1°C and a low of -14.7°C. The variability in temperature was greater for air temperature readings than those taken at the critical zones; there were lower diurnal fluctuations and a reduced overall range of temperature throughout measured period at the critical zone compared to air temperatures (Figure 3.3.).

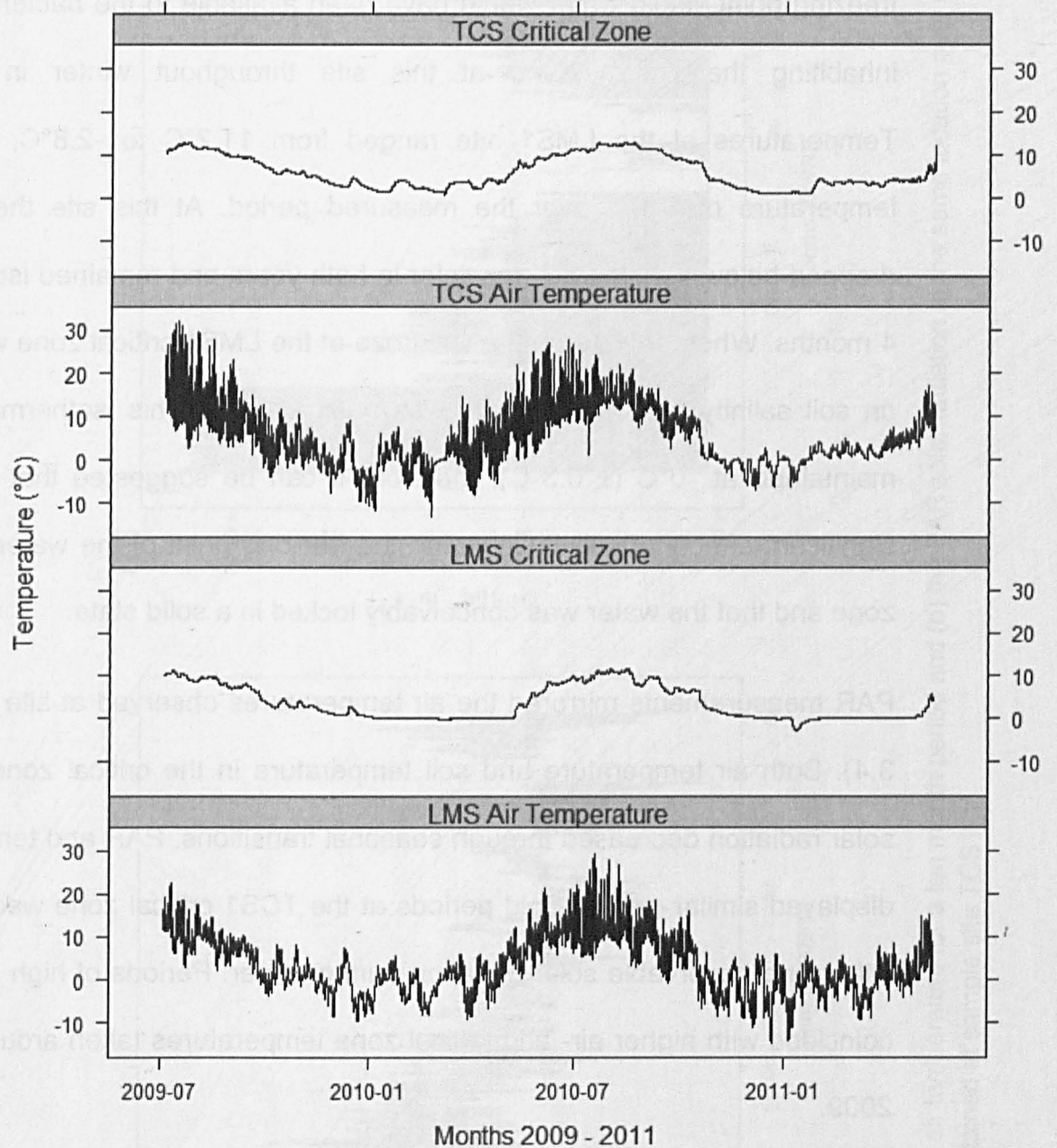


Figure 3.3: Critical zone and air temperature data from sites LMS1 and TCS1. Data collected over a 23 month period from June 2009.

Temperatures at the TCS1 critical zone ranged from 13.1°C to 1.0°C with a mean temperature of 6.2°C over the period of study. As temperatures never reached freezing point, liquid water would have been available to the bacterial community inhabiting the critical zone at this site throughout winter in both years. Temperatures at the LMS1 site ranged from 11.2°C to -2.8°C, with a mean temperature of 3.4°C over the measured period. At this site the temperature dropped below freezing during winter in both years and remained isothermal for c. 4 months. Whether water in the soil froze at the LMS1 critical zone was depended on soil salinity; although salinity was not recorded. This isothermal phase was maintained at 0°C ($\pm 0.3^\circ\text{C}$), therefore it can be suggested that there was no significant effects of salinity lowering the freezing point of the water at the critical zone and that the water was conceivably locked in a solid state.

PAR measurements mirrored the air temperatures observed at site TCS1 (Figure 3.4). Both air temperature and soil temperature in the critical zone, declined as solar radiation decreased through seasonal transitions. PAR and temperature also displayed similar peaks. Cold periods at the TCS1 critical zone were matched by little or no observable solar radiation during winter. Periods of high solar radiation coincided with higher air- and critical zone temperatures taken around September 2009.

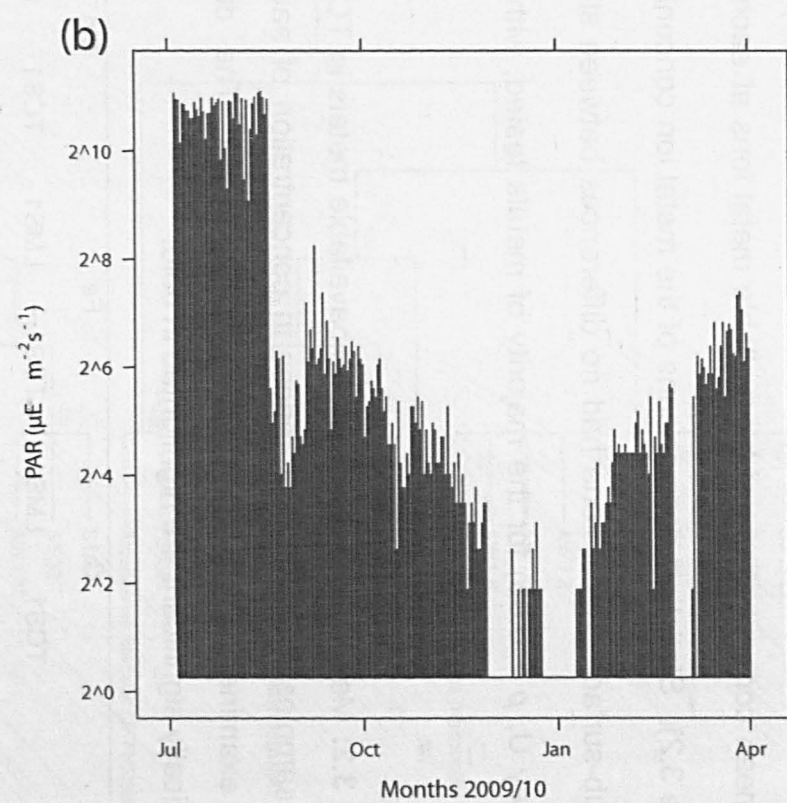
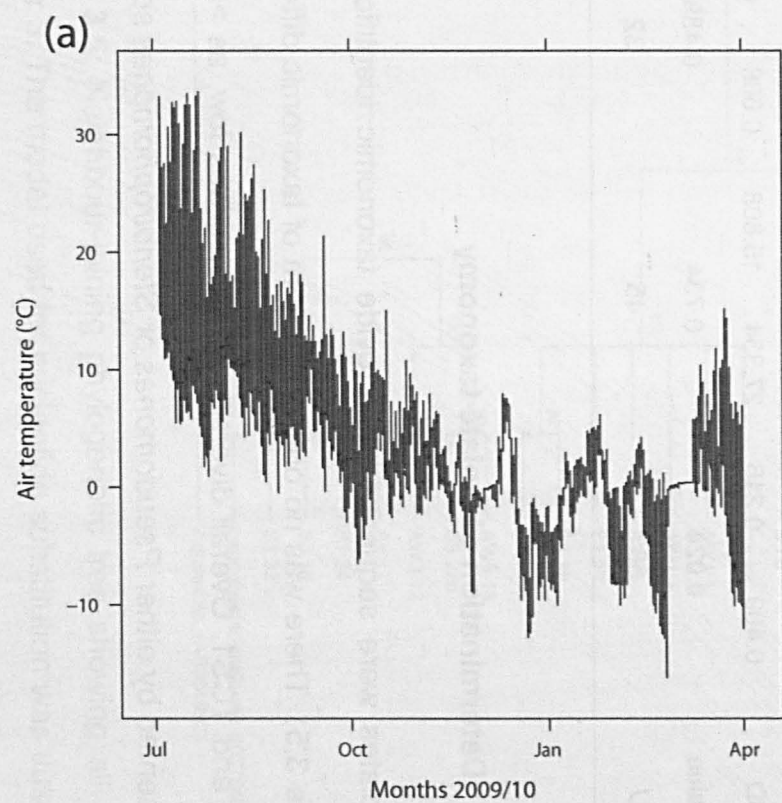


Figure 3.4: (a) air temperature for a ten month period and (b) the PAR solar radiation at the same location over equal time period. Data obtained at sample site TCS1.

3.3.2. Bioavailable metals at the critical zone

The mean concentrations of bioavailable metal ions at each site were calculated (Table 3.2). Statistical comparisons of the metal ion concentrations indicated that the sub-surface critical zone had no differences between sites (Table 3.2; Mann Whitney U, $p > 0.05$) for the majority of metals tested, with the exception of Cu ions.

Table 3.2: Mean concentrations of bioavailable metals in TCS1 and LMS1 critical zone samples ($n = 18$). Differences in concentration of each bioavailable metal were examined using a Mann Whitney U test. The differences that were statistically significant are highlighted in bold.

	Cu		Fe		Mn		Zn	
	TCS1	LMS1	TCS1	LMS1	TCS1	LMS1	TCS1	LMS1
Mean (mM)	1.128	0.738	44.185	42.823	1.277	1.574	0.264	0.217
SD	0.409	0.216	27.334	15.808	0.688	0.529	0.176	0.208
<i>p</i> value	0.024		0.734		0.489		0.135	
<i>U</i>	66		45		32		58	

3.3.3. Determination of isolate taxonomy

All isolates were sequenced to provide taxonomic identification to genus level (Figure 3.5). There was no obvious pattern of taxonomic difference between sites LMS1 and TCS1. Overall diversity of isolates was low, as >50% of isolates were represented by either *Pseudomonas* or *Stenotrophomonas* spp.

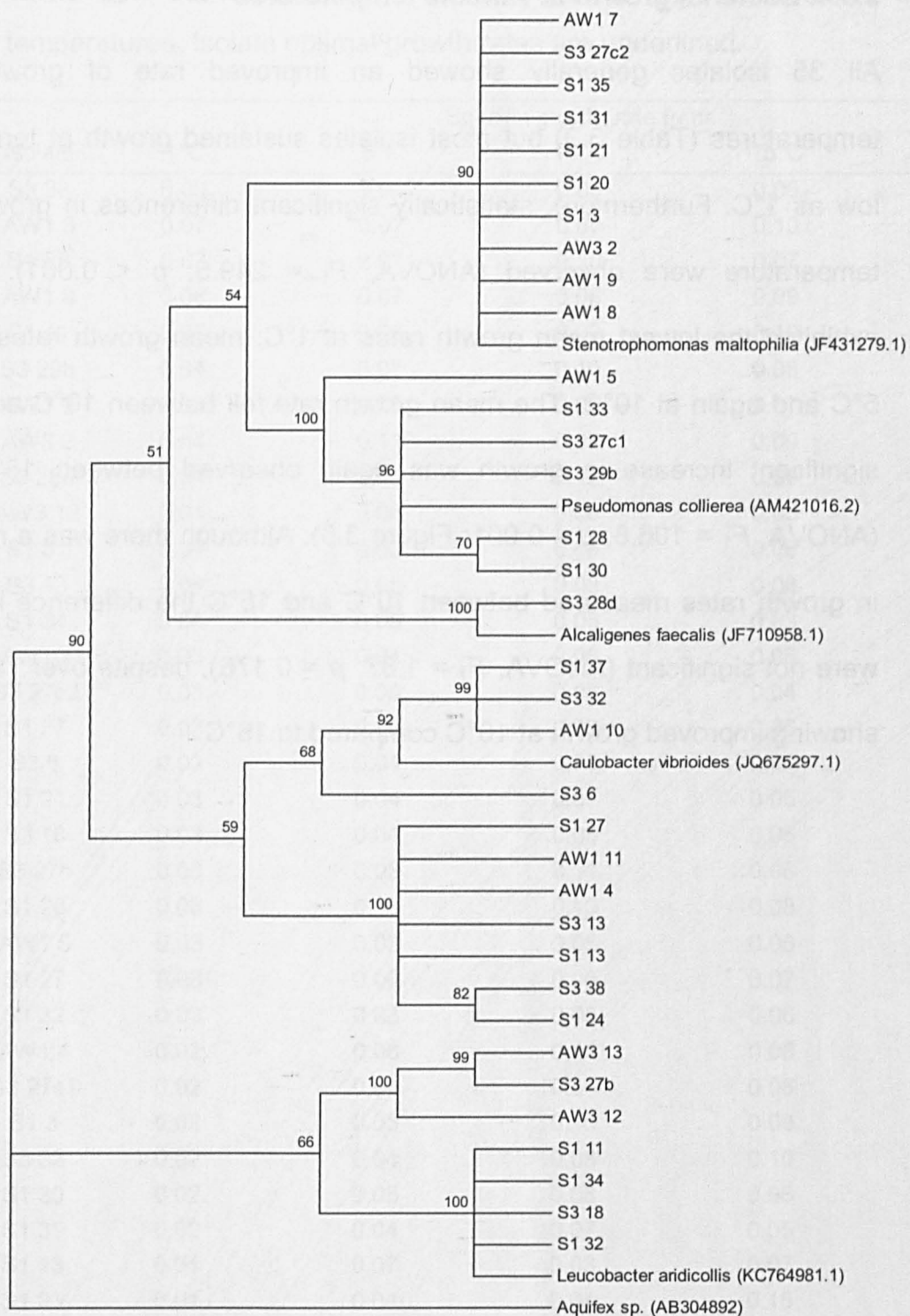


Figure 3.5: Neighbour-joining phylogenetic tree showing all 35 isolates used in Chapter 3. The model used for nucleotide substitution was Jukes-Cantor (Jukes et al., 1969). All branches of tree show bootstrap values >0.5.

3.3.4. Bacterial growth at variable temperatures

All 35 isolates generally showed an improved rate of growth at higher temperatures (Table 3.3) but most isolates sustained growth at temperatures as low as 1°C. Furthermore, statistically significant differences in growth rates with temperature were observed (ANOVA, $F_{1,4} = 249.5$, $p < 0.001$). The isolates exhibited the lowest mean growth rates at 1°C; mean growth rates increased at 5°C and again at 10°C. The mean growth rate fell between 10°C and 15°C yet a significant increase in growth was again observed between 15°C and 22°C (ANOVA, $F_1 = 106.8$, $p < 0.001$; Figure 3.6). Although there was a negative trend in growth rates measured between 10°C and 15°C the difference in growth rate were not significant (ANOVA, $F_1 = 1.87$, $p = 0.176$), despite over 76% of isolates showing improved growth at 10°C compared to 15°C.

Table 3.3: Individual isolates specific growth rates at different experimental temperatures. Isolate optimal growth rates are underlined.

Isolate	Specific growth rate (μ hr ⁻¹)				
	1°C	5°C	10°C	15°C	22°C
S3 38	0.09	0.04	0.08	0.05	<u>0.24</u>
AW1 3	0.07	0.07	0.07	0.10	<u>0.22</u>
S1 28	0.07	0.07	0.10	0.07	<u>0.19</u>
AW1 8	0.06	0.07	0.06	0.09	<u>0.17</u>
S1 11	0.05	0.08	0.09	0.10	<u>0.18</u>
S3 29b	0.04	0.07	0.10	0.08	<u>0.18</u>
AW1 9	0.04	0.06	0.09	0.07	<u>0.22</u>
AW3 2	0.04	0.11	0.07	0.09	<u>0.16</u>
S3 28d	0.04	0.03	0.12	0.07	<u>0.17</u>
AW3 12	0.04	0.08	0.08	0.05	<u>0.18</u>
S1 31	0.04	0.03	0.08	0.06	<u>0.18</u>
S3 13	0.04	0.06	0.08	0.06	<u>0.20</u>
S1 34	0.04	0.05	0.06	0.03	<u>0.14</u>
AW1 7	0.03	0.04	0.06	0.05	<u>0.19</u>
S3 27c2	0.03	0.05	0.05	0.04	<u>0.17</u>
S1 37	0.03	0.08	0.07	0.06	<u>0.13</u>
S3 6	0.03	0.04	0.06	0.05	<u>0.18</u>
S1 21	0.03	0.04	0.07	0.05	<u>0.13</u>
S3 18	0.03	0.04	0.05	0.05	<u>0.22</u>
S3 27b	0.03	0.03	0.11	0.06	<u>0.19</u>
S1 20	0.03	0.03	<u>0.13</u>	0.08	<u>0.13</u>
AW1 5	0.03	0.03	0.05	0.05	<u>0.06</u>
S1 27	0.03	0.06	0.06	0.07	<u>0.20</u>
S1 32	0.03	0.03	0.08	0.06	<u>0.16</u>
AW1 4	0.02	0.06	0.11	0.08	<u>0.18</u>
S3 27c1	0.02	0.04	0.05	0.06	<u>0.22</u>
S1 3	0.02	0.05	0.10	0.09	<u>0.11</u>
S3 32	0.02	0.04	0.08	0.10	<u>0.15</u>
S1 30	0.02	0.05	0.08	0.05	<u>0.20</u>
S1 35	0.02	0.04	0.07	0.05	<u>0.12</u>
S1 13	0.01	0.07	0.03	0.07	<u>0.20</u>
S1 33	0.01	0.04	0.04	0.15	<u>0.20</u>
AW1 10	0.01	0.03	0.06	0.05	<u>0.20</u>
S1 24	0.00	0.00	<u>0.08</u>	0.05	0.07
AW3 13	0.00	0.04	0.09	0.08	<u>0.19</u>
AW1 11	0.00	0.01	0.04	<u>0.06</u>	0.05

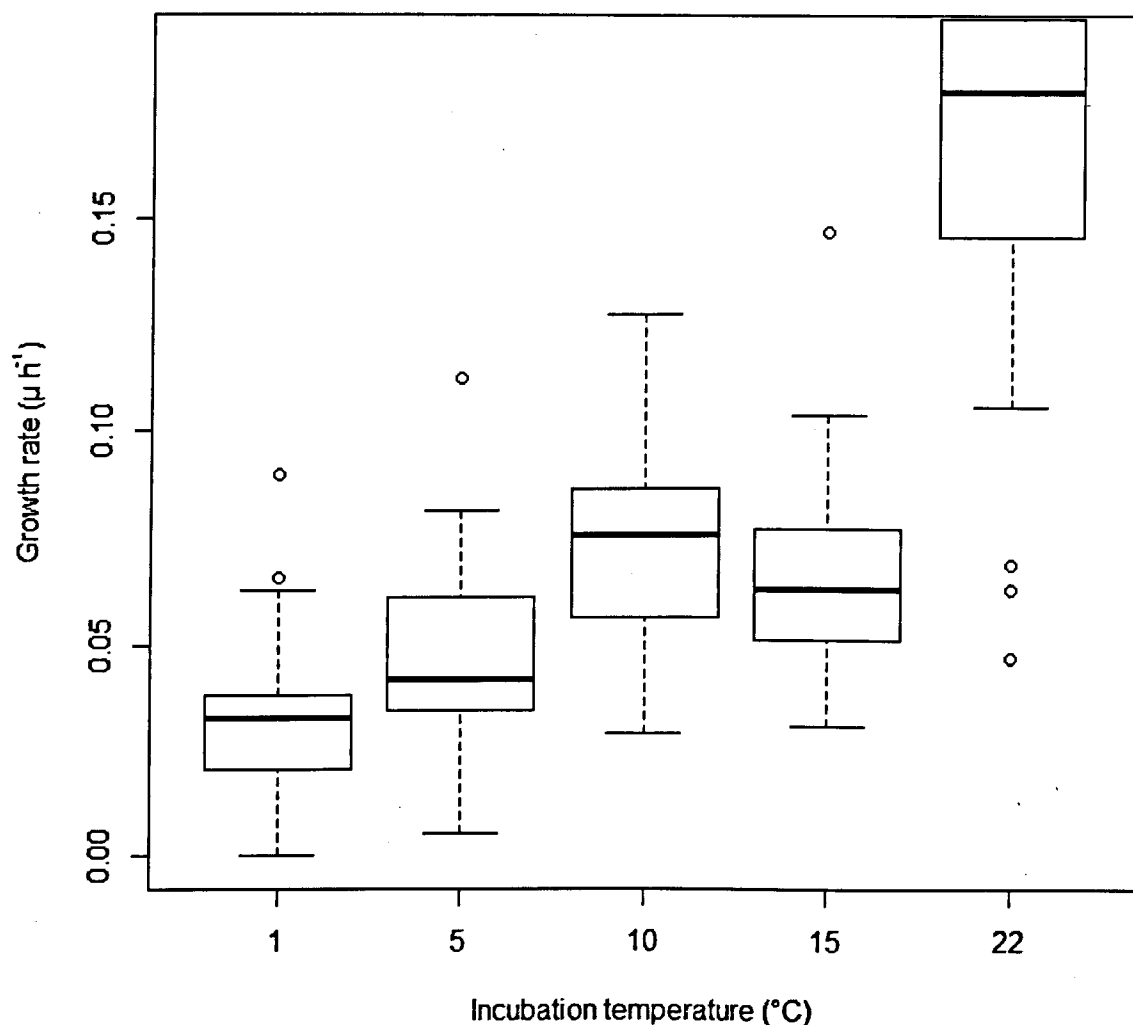


Figure 3.6: Box and whisker plot indicating the median growth rates of all bacterial isolates at five different temperatures. Whiskers represent 95% confidence intervals.

3.3.5. Mineral phosphate solubilisation cultures

Phenotypic tests for phosphate solubilisation of recovered isolates indicated halos were present around several of the bacterial strains inoculated onto the MPS plates (Figure 3.7).

Thirty two of the 35 bacterial isolates tested exhibited evidence of biotic induced weathering, the three isolates that did not exhibit the MPS phenotype originated from site LMS1. Of the three bacterial isolates that showed no MPS phenotype (S1 32, *Leucobacter* spp; S1 33, *Pseudomonas* spp; AW1 11, *Paenibacillus* spp), two were cultured using treatments A (S1 32 & S1 33) and one using treatment C (AW1 11; see Table 3.1 for details of each treatment).

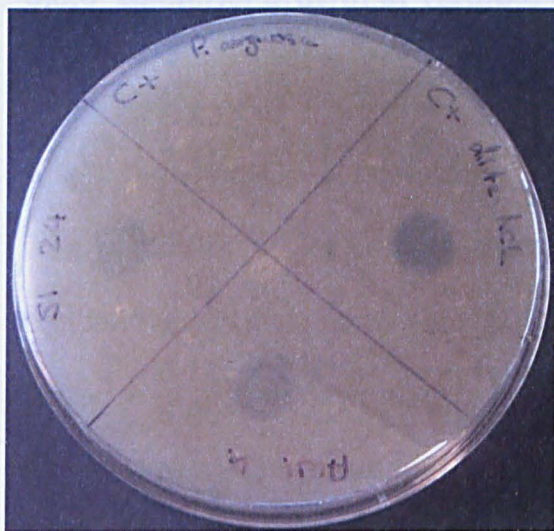


Figure 3.7: Weathering capabilities of bacterial isolates. Image shows the development of a halo following solubilisation of TCA when bacterial inoculants (Table 3.4) were inoculated onto media. A strong halo is present in the right quadrant of the MPS agar plate inoculated with 5% HCl control. Weaker halos observed on the left and bottom quadrants were created by bacterial isolates (*Arthrobacter* spp and *Brevundimonas* spp respectively). No halo was observed in the top quadrant; this was the failed bacterial positive control *P. aeruginosa*. Negative control was an equal volume of sdH₂O containing no cells.

The measurements of the halo areas provided a quantifiable measure of the weathering capability of each isolate, given in Table 3.4. Specifically, it was observed that there was no significant difference in the bacterial community weathering capability from either sample site (Mann-Whitney U test, $U = 153$, $p = 0.7456$) nor dominance of a particular genus amongst the most efficient MPS Bacteria (Kruskal-Wallis test, $df = 9$, $p = 0.67$).

Table 3.4: Weathering capability of individual isolates grown on MPS plates. Displayed are the: taxonomic affiliation, area of the halo and original isolation treatments for each isolate.

Isolate #	Genus	Area (mm ²)	Treatment
S1 37	<i>Brevundimonas spp</i>	33.3	A
S1 34	<i>Leucobacter spp</i>	63.1	A
S1 32	<i>Leucobacter spp</i>	0	A
S1 27	<i>Leucobacter spp</i>	101	A
S1 33	<i>Pseudomonas spp</i>	0	A
S1 28	<i>Pseudomonas spp</i>	36.9	A
S1 30	<i>Pseudomonas spp</i>	80.6	A
S1 31	<i>Stenotrophomonas spp</i>	67.1	A
S1 35	<i>Stenotrophomonas spp</i>	111	A
S1 24	<i>Arthrobacter spp</i>	68.3	B
S1 13	<i>Leucobacter spp</i>	47.8	B
S1 11	<i>Leucobacter spp</i>	110.5	B
S1 3	<i>Stenotrophomonas spp</i>	82.3	B
S1 21	<i>Stenotrophomonas spp</i>	29.5	B
S1 20	<i>Stenotrophomonas spp</i>	34.4	B
AW1 10	<i>Brevundimonas spp</i>	91.6	C
AW1 4	<i>Brevundimonas spp</i>	99.8	C
AW1 11	<i>Paenibacillus spp</i>	0	C
AW1 5	<i>Pseudomonas spp</i>	69.3	C
AW1 9	<i>Stenotrophomonas spp</i>	61.9	C
AW1 8	<i>Stenotrophomonas spp</i>	65	C
AW1 7	<i>Stenotrophomonas spp</i>	96.2	C
S3 28d	<i>Alcaligenes spp</i>	85.3	D
S3 32	<i>Brevundimonas spp</i>	82.4	D
S3 27c1	<i>Pseudomonas spp</i>	46.7	D
S3 38	<i>Pseudomonas spp</i>	10.5	D
S3 29b	<i>Pseudomonas spp</i>	42.6	D
S3 27b	<i>Staphylococcus spp</i>	61.1	D
S3 27c2	<i>Stenotrophomonas spp</i>	75.3	D
S3 6	<i>Ochrobactrum spp</i>	79.6	E
S3 18	<i>Stenotrophomonas spp</i>	74.3	E
S3 13	<i>Stenotrophomonas spp</i>	76.4	E
AW3 13	<i>Bacillus spp</i>	46.9	F
AW3 12	<i>Paenibacillus spp</i>	94.3	F
AW3 2	<i>Stenotrophomonas spp</i>	87.2	F

Furthermore, the initial culturing treatments (A-F) had no significant effect on the weathering capability of bacterial isolates tested (Kruskal-Wallis test, $df = 5$, $p = 0.8$) nor the temperatures employed (Mann-Whitney U, $U = 188$, $p = 0.239$). Finally, the weathering capability of bacterial isolates did not differ significantly between initial aeration treatments during isolation (Mann-Whitney U, $U = 93$, $p = 0.249$).

3.3.6. Bacterial growth at variable Cu concentrations

The bacteria selected for Cu treatment exhibited a reduction in mean growth rate with increasing Cu concentration (Figure 3.8). A significant reduction in growth was observed at concentrations of 1 mM Cu when compared to 0.1 mM Cu concentrations treatments (ANOVA, $F_1 = 4.606$, $p = 0.037$). There was a complete absence of growth when Cu concentrations were 10 mM or higher (Table 3.5) and there were no measureable differences between biotic treatments from these higher Cu concentrations (10 and 100 mM) and abiotic controls as all treatments showed zero growth. At the lowest concentrations of Cu, the overall growth rates were significantly lower than those observed during 22°C temperature treatments that contain zero addition Cu within the growth media (Mann Whitney U, $U = 403$ $p < 0.005$).

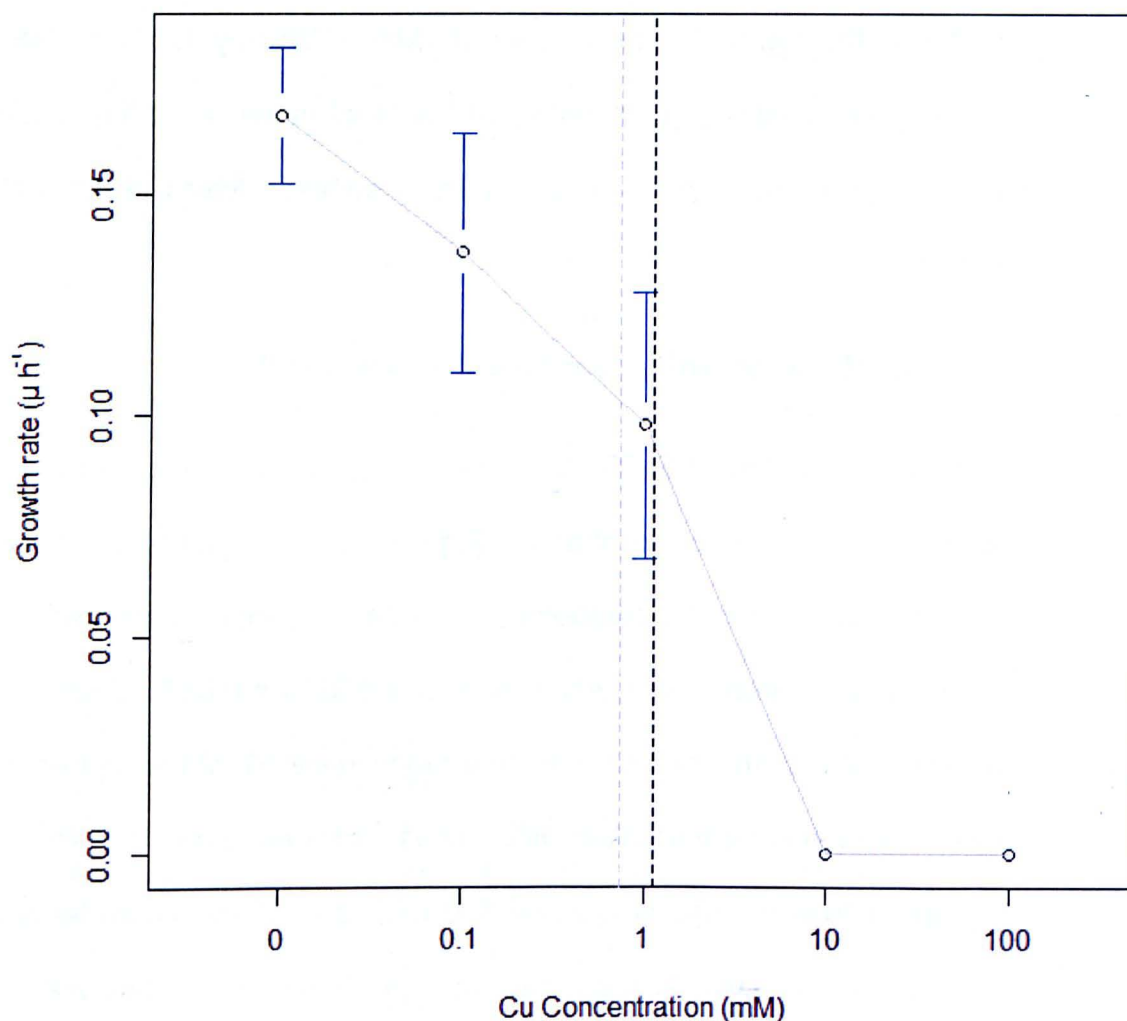


Figure 3.8: Mean growth rates of isolates grown at five experimental Cu concentrations (error bars indicate 95% CI). Grey and black vertical lines depict minimum and maximum Cu concentrations, respectively, measured by ICP-AES from the critical zone. X axis is presented in a logarithmic scale.

The Cu concentration at which bacterial growth ceased was between 1 and 10 mM. The maximum bioavailable Cu measured at the critical zone (3.0 mM) lay well within this range. Moreover, concentrations as low as 0.1 mM had a detrimental

effect on bacterial growth, which was substantially lower than the concentrations of Cu measured in the environmental samples.

Table 3.5: Growth rates of each isolate grown under various Cu concentration additions.

Isolate #	Growth rate ($\mu \text{ hr}^{-1}$)				
	Cu 0.0 mM	Cu 0.1 mM	Cu 1.0 mM	Cu 10 mM	Cu 100 mM
AW1 11	0.047	0.035	-0.001	0.000	0.000
S3 13	0.205	0.103	-0.001	0.000	0.000
S3 6	0.183	0.107	0.123	0.000	0.000
S1 37	0.131	0.110	0.118	0.000	0.000
S1 24	0.069	0.115	0.000	0.000	0.000
S3 28d	0.165	0.118	0.061	0.000	0.000
S1 32	0.162	0.128	0.119	0.000	0.000
S3 32	0.151	0.130	0.110	0.000	0.000
S1 3	0.106	0.131	0.132	0.000	0.000
S1 13	0.196	0.145	0.125	0.000	0.000
S3 29b	0.181	0.159	0.129	0.000	0.000
AW3 13	0.187	0.166	0.125	0.000	0.000
S1 21	0.130	0.167	0.147	0.000	0.000
S1 33	0.198	0.184	0.158	0.000	0.000
AW3 12	0.183	0.259	0.124	0.000	0.000

3.3.7. Glucose dehydrogenase gene isolation

The expected length of the *GDH* gene was 900 b.p. (Shigematsu et al., 2005), however the PCR products obtained showed a high level of non-specific products ranging from c. 400 bases to over 1000 bases in length (Figure 3.9). None of the bacterial isolates subjected to the *GDH* gene assay provided any PCR amplicons c. 900 bases in length.

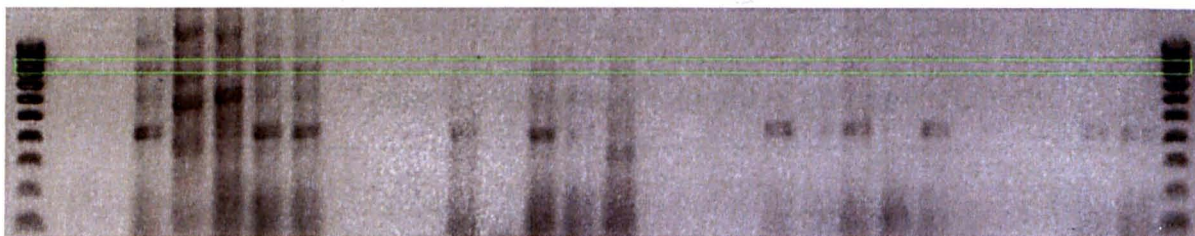


Figure 3.9: Electrophoresis gel of *GDH* gene PCR assay. The area highlighted green signifies the 900 n.t. PCR product expected. All lanes were used and harbour amplicons of randomly selected isolates, not all isolates provided PCR products and those isolates that did gave many non-specific bands. Hyperladder IV (Bioline, London, UK) was used for amplicon length comparison.

Not all isolates that were recorded as possessing the MPS phenotype (Table 3.4) yielded PCR products using the *GDH* gene assay. Of the 56 isolates that were included in the *GDH* gene assay, only 68% of known MPS phenotypes showed PCR products, of any length. Of the three isolates that were previously reported as not exhibiting the MPS phenotype, one provided a PCR product of c. 400 bases in length.

3.3.8. *GDH* gene PCR optimization and identification

It was apparent that non-specific binding had made all results from *GDH* functional gene assay non-viable. In order to assess the effect of different PCR conditions on amplicon size an optimised temperature gradient PCR was carried out using an isolate that produced amplicons of three distinct lengths: c. 400, c. 600 and c. 1000 nucleotide bases (Figure 3.10). This assay revealed the optimal annealing temperature to be 60.4°C (Lane 9 in Figure 3.10), which was 10.4°C higher than the temperature stated in the literature and provided more specific products than the published *GDH* gene assay protocol (Shigematsu et al., 2005).

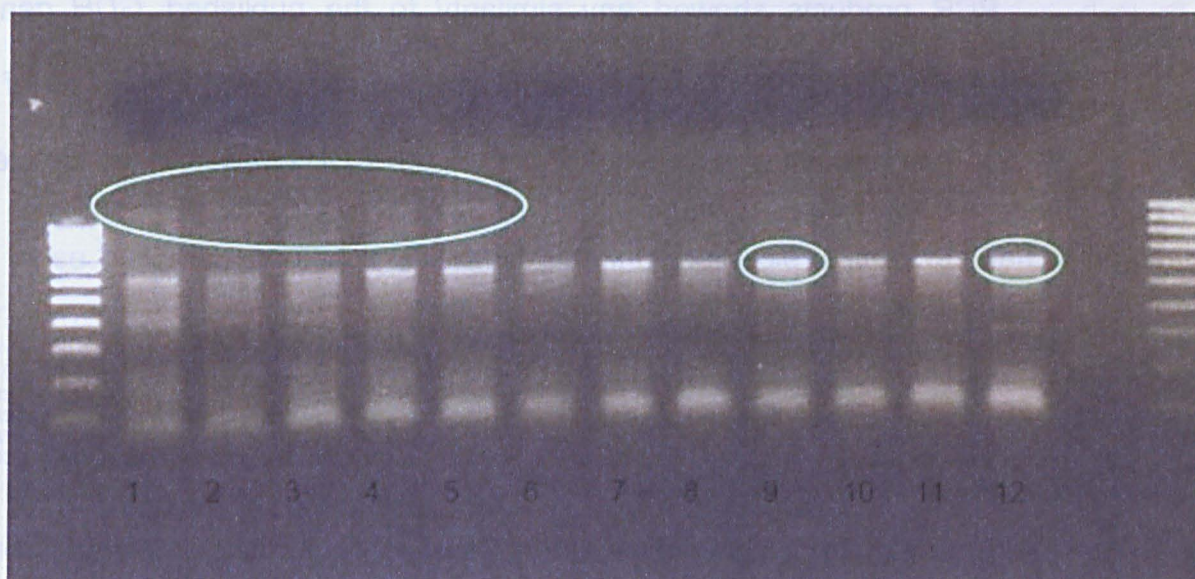


Figure 3.10: Agarose gel of *GDH* PCR products achieved using isolate AW1 8, amplified using a temperature gradient PCR. Individual bands and those pooled for subsequent Sanger sequencing are highlighted by green ovals. Temperatures in lane 1 were 50.1°C rising incrementally (1.38°C per lane) to lane 12 which had a temperature of 65.3°C. Hyperladder IV (Bioline, London, UK) is used for amplicon length comparison.

Given the discrepancy between amplicon length published by Shigematsu et al. (2005) and the amplicon lengths obtained in this study, identification of the *GDH* gene's nucleotide composition was conducted by Sanger sequencing. Concentrations of amplified PCR products of c. 1000 bases from the first 5 lanes were too low for sequencing, therefore a pooled sample of all five was sequenced. However, the products showed no identifiable individual nucleotides, indicating that all five sequences were likely non-specific.

Sequencing of PCR products from lanes 9 and 12 of the gel provided two copies of c. 600 base amplicons. These fragments were sequenced separately and

compared to published sequences of *GDH* gene (Figure 3.11). Neither of the two PCR products showed any similarity to the published *GDH* gene sequences (EMBL AAK98943.1) nor to each other. This indicated that, while of equal length, neither product was an amplification of the *GDH* gene from the isolate.

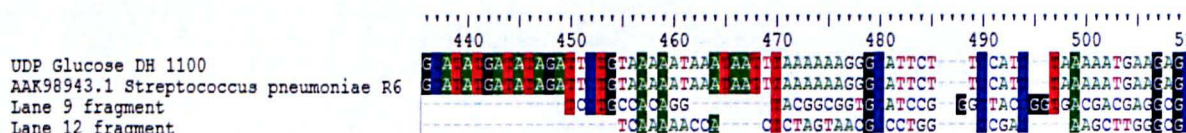


Figure 3.11: Nucleotide sequences of PCR products obtained from *GDH* gene assay compared to publish versions of *GDH* gene. Highlighted columns indicate that >75% similarity of base between sequences.

3.4. Discussion

This chapter investigates the physiological interactions between biotic and abiotic environmental conditions that may be experienced by bacteria inhabiting the critical zone, and whether these conditions were optimal to bacterial growth. For those bacteria adapted to this environment, their role in the weathering process was investigated using their ability to weather insoluble mineral state phosphate as a proxy for weathering in the environment.

3.4.1. Environmental conditions

Both LMS and TCS sites exhibited large temperature ranges throughout the year. The critical zone and air temperatures at site LMS1 were lower than at site TCS1, despite the fact that temperatures were measured at the same depth and elevation, respectively (Figure 3.3). This difference in temperature may have been due to differences in the solar radiation experienced at each site. Site TCS1 faces south-south east and will consequently have higher solar radiation throughout summer and autumn seasons, whereas site LMS1 faces east-north east. Both sample sites are located in moderate valleys and sample areas will be shaded at certain times of the year. However during summer months, when the sun is highest, site TCS1 experiences 24 hours of solar exposure, meteorological conditions permitting. Conversely, site LMS1 experiences shade during late afternoon as the valley side casts a shadow over the sample site. Measurement of solar radiation at site LMS1 was not conducted due to technical failure of the equipment.

Solar PAR readings exhibited highly similar patterns of fluctuations to those observed in the air temperature of TCS1 (Figure 3.4). The coldest period

experienced at the soil surface coincided with an almost complete lack of solar radiation recorded during the December-January period. This pattern was observed for each of the cold periods recorded. Conversely, high temperatures observed also coincided with increases in solar radiation. For example, the peak in temperatures recorded during September 2009 (Figure 3.4). The effects of increasing solar radiation on increasing soil temperatures have been well documented (Bristow & Campbell, 1984, Fitter et al., 1998).

Generally, temperature ranges at the critical zone were narrower than those at the soil surface and the exhibited fluctuations of the LMS site were broader at the TCS site (14.4°C and 12.1°C respectively). Seasonal warm periods exhibited similar temperature ranges; however, seasonal cold periods were markedly different as while the TCS site critical zone was measured as fluctuating by c. 3°C the LMS site remained isothermal at 0°C. This can be attributed to the differing thermal inertia of the soils (Lawrence & Slater, 2008, Smith et al., 1997) which depends on the SOC content of the soils. Since the TCS site contains higher concentrations of SOC (Chapter II), it may also have an increased thermal insulation capability, restricting thermal energy transfer between the soil and air. The presence of higher plants at the TCS site had the potential to further insulate the critical zone through deposition of plant thatch (Smith et al., 1997), which may limit the variation of temperature at the TCS site; the LMS site had no observed development of these thermal insulation effects. However, the presence of plants and the associated root systems can confound these insulative properties. The plant itself can act as a heat sink transferring energy between the soil and the air; however, this assumes the presence of liquid water within the plant and is dependent on

surface area of each plant (Wu et al., 2007). Neither of these variables were measured here, therefore no estimation of this effect can be made.

3.4.2. The effect of temperature on bacterial growth

A wide range of culturable bacterial isolates were obtained from the cold critical zone studied here. In microcosm experiments, however, most isolates grew optimally at 22°C and not at any temperature that was experienced at the critical zone of LMS or TCS sites. Only isolates AW1 11 (*Paenibacillus spp*), S1 24 (*Arthrobacter spp*) and S1 20 (*Stenotrophomonas spp*) exhibited optimal growth at temperatures equal to or lower than experienced at the LMS and TCS sites (10 – 15°C). Therefore, these isolates can be described as psychrotolerant. However, they cannot be described as psychrophilic since all exhibited some growth at temperatures greater than 20°C, a temperature which is greater than experienced at the sub-surface critical zone. Clearly then, the majority of the bacterial community examined here, with some exceptions, do not inhabit a region suitable for their optimal growth (H4).

The highest temperatures experienced at the critical zone were lower than the optimal temperatures for growth of most isolates. Hence, it is evident that the critical zone is an extreme environment and many of the bacteria inhabiting this area also fall into the category of extremophiles/extremotrophs, as defined by Mueller et al. (2005). Given the harsh environment that these bacteria inhabit, two questions can be postulated:

- 1) Why do these bacteria persist in an extreme environment?
- 2) Why are these bacteria not out-competed by microbes with growth optima better adapted to the environment?

Tang et al. (1997) presented evidence that the optimum growth temperature for cyanobacteria in polar regions was higher than the environmental temperatures, similar to this study. They propose that these organisms subsist by slow persistent growth and that other competitive advantages, such as adaptation to desiccation and freeze tolerance, might account for their dominance. Similarly, Cockell et al. (2010) studied bacterial isolates (primarily *Arthrobacter*) living in gypsum in the Canadian High Arctic. They also found no psychrophiles but suggested desiccation and freeze-tolerance might explain the presence of psychrotolerant bacteria living in high arctic soils and salts. Applying these principles to this study, periodic desiccation cannot be discounted, at least at the LMS site, as temperatures could fall so low that freezing occurs. Other competitive advantages might be oligotrophy and mineral weathering capacity, such as is discussed in chapters II and this chapter respectively. For example, oligotrophy can offer competitive advantages for adapted organisms, permitting these individuals to competitively exclude other organisms from limited nutrient pools, potentially generated by bacterial weathering of phosphate.

3.4.3. Metal toxicity

Microcosm experiments show that bacterial growth was inhibited at even the lowest concentrations of Cu supplied, which were lower than bio-available Cu concentrations at the LMS and TCS sites. Therefore it would be expected that the levels of Cu in these sites would be sufficient to impede bacterial growth *in-situ* (H4). This suggests that Cu is acting as a stressor on the bacterial community and that there is potential for lethal concentrations to limit bacterial growth to be present at the critical zone.

Further experiments using a higher resolution of Cu concentrations between 1 mM and 10 mM could determine more precisely the concentration of Cu at which bacterial growth ceases in communities from the LMS and TCS sites. This subsequent data could indicate if the lethal limits to bacterial growth are available in the environment from which they inhabit. Data obtained in this study (XRF and ICP-AES, Chapters II and III, respectively) indicates that the maximum mean soil Cu concentrations are c. 1.17 mM, which does impede growth but may not be sufficient to cause a cessation of growth. However, it should be noted that persistent exposure to Cu and other heavy metals may increase bacterial tolerance to heavy metals through elimination of less tolerant individuals in the community (Diaz-Ravina & Baath, 1996).

The use of sterile crushed soil to enrich the organics within the bacterial isolation stage of this chapter may have skewed the results of the Cu growth trials. Following completion of this experiment it was postulated (Uroz, 2013 per. Comm.) that the sterile soils, while offering a potential source of nutrients and organics, may have leached Cu from the soil particles to the agar on which the bacterial isolates were grown. Using the known concentrations of Cu in this Icelandic soil type (Table 3.2) it was calculated that all isolates may have been grown and sub-cultured in Cu concentrations of c. 0.043 mM Cu (Figure 3.12). This may have resulted in a cultured community more resistant to Cu than was likely from growth media that was without the added sterile crushed soils. That said, this resulted in the growth conditions during isolation being closer to the environment than originally intended. Furthermore, it may add more environmental significance to the poor growth on Cu enriched media that was recorded for the bacterial isolates in this chapter. Given that this study may have inadvertently selected for Cu

tolerant bacteria, the poor growth of bacterial isolates recorded at experimental Cu concentrations may infer that the bacteria from the sub-surface critical zone that do not possess Cu tolerance are even more susceptible to the high environmental Cu concentrations that is apparent from these data.

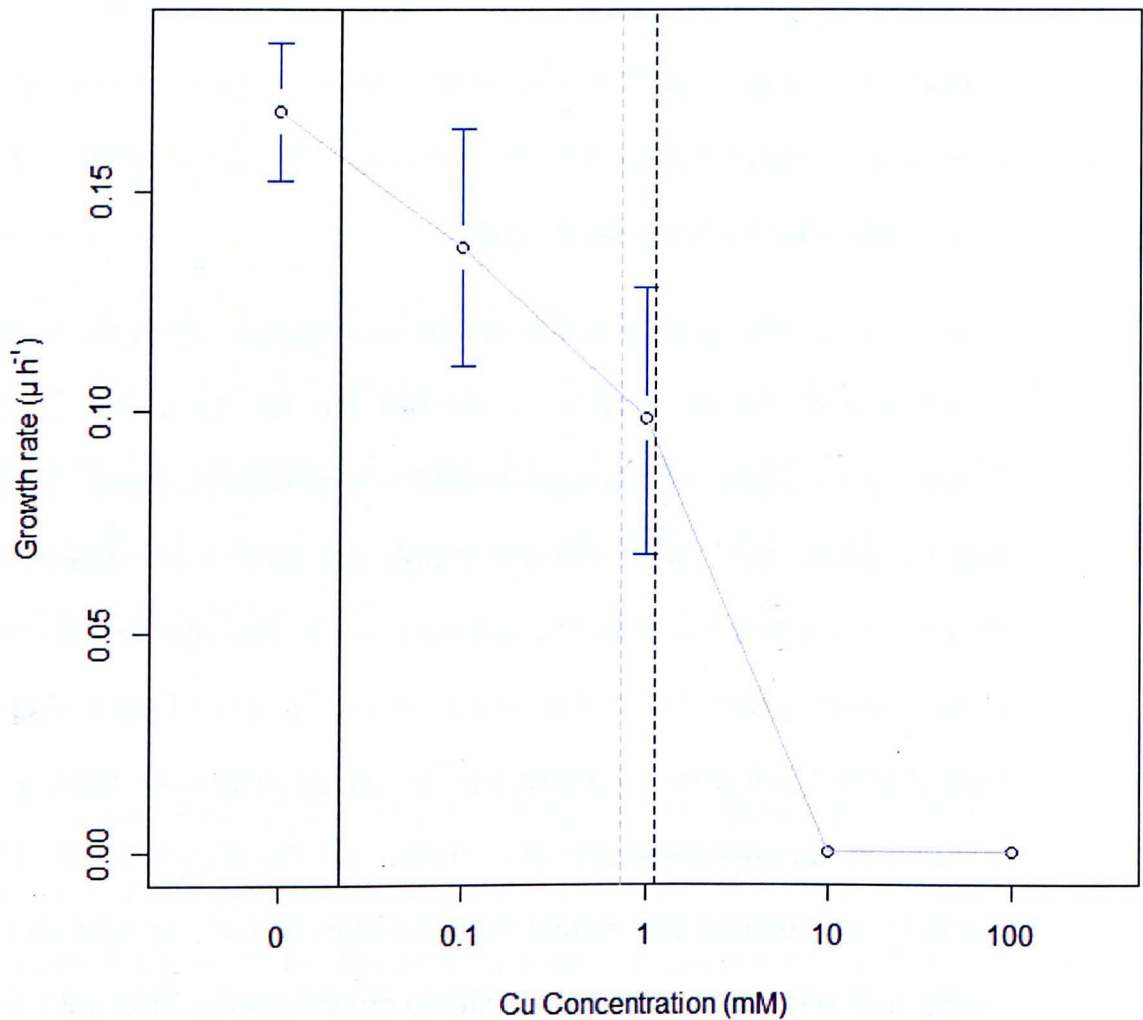


Figure 3.12: The growth decline of the bacterial isolates in various Cu concentrations of growth media. Added here is the level of Cu expected in the bacterial isolation plates (Section 3.2.5.), this can be seen as the vertical solid line.

3.4.4. Bacterial induced phosphate solubilisation

Some volcanic regions are oligotrophic (Cockell et al., 2011, Jorquera et al., 2008) due to a limitation in bioavailable phosphate. Many bacterial species are known to thrive in phosphate-limited environments by obtaining the phosphorus required for growth through weathering and in the process they release insoluble phosphate into the environment (Goldstein et al., 1999, Hamdali et al., 2012, Jorquera et al., 2008, Perez et al., 2007, Richardson, 2001, Vera et al., 2008).

This highlights the importance of bacteria at the critical zone for soil fertility, as these organisms are in direct contact with vast sources of insoluble phosphate that are required for life in the local ecosystem. This can be liberated and made bio-available through hydrolysis of the parent material.

It is clear that most of the bacteria isolated in this study were capable of solubilising mineral state phosphate. However no single taxon exhibited a clearly greater ability to solubilise phosphate over any other (H5). Bacterial isolates of *Gammaproteobacteria* (S1 35) and *Actinobacteria* (S1 11) displayed the highest MPS ability but this was not consistent across all isolates of the same bacterial class. In general, it appears that most isolates displayed some ability to weather mineral phosphate and, as a community, bacterial activity would contribute substantially to weathering at the critical zone.

Bacterial weathering appeared to be a generalist function that crossed many phylogenetic groups. Furthermore, there was no evidence of enhanced phosphate weathering capability by the bacterial community from either the LMS or the TCS sites (H6). Therefore, the different plant communities at each site (LMS and TCS) were not recorded as adding a selective pressure on to the bacterial community,

designed to promote the MPS phenotype. This infers a bacterial community that possess a high level of functional redundancy for phosphate weathering. If this is so, the importance of bacteria in the weathering process may be underestimated as extreme environments such as the one studied here (Section 3.4.2) are believed to harbour lower levels of functional redundancy (Wall & Virginia, 1999). Assuming that the bacterial enhanced weathering studied here is a true generalist processes then this phenotype may be of global significance.

3.4.5. Mechanisms for phosphate mineral weathering

In this study, MPS phenotypes were observed in a bioassay using glucose as the sole carbon source, making gluconic acid the organic acid most likely to be produced. In order to assess the role of gluconic acid production in weathering, assays were established to isolate the *GDH* functional gene. The first assay using 28 isolates produced initially encouraging results, as all visible products were from isolates with the MPS phenotype. Although, there were a number of false negatives, no false positives were detected, but, the product length did not match the gene length given in the literature. This may have been an optimization error or because the primer set was not specific enough to target isolates from all taxa. Further investigation of a larger number of samples ($n = 56$) was less successful, with a mixture of false negatives and false positives; the lengths of all products from the second trial differed from the target gene length by c. 500 bases. The primer set appeared to have targeted two conserved sites encompassing a variable region rather than the *GDH* functional gene.

There are several possibilities why this assay failed to isolate the *GDH* gene from bacterial isolates. The positive controls used in Shigematsu et al. (2005) were not

used in this study, due to financial constraints. Thus, although the bioassay (Section 3.2.8.) successfully identified MPS phenotypes, it is possible that none of the 56 isolates examined in this molecular study possessed the *GDH* functional gene.

During the initial phases of this research and its inception, the production of gluconic acid remained the most parsimonious explanation for the positive results of the MPS bioassay, as the isolates were able to solubilise mineral phosphate when supplied with glucose as their sole carbon source. However, upon completion of this section of the project it emerged that the initial assumption that glucose could be metabolised using a biochemical pathway containing the *GDH* gene was an over simplification (Murrell, 2013, per. comm.). While it was feasible that this process is occurring, as gluconic acid is a by-product of prokaryotic glucose metabolism (Ramachandran et al., 2006), the potential biochemical products that could emerge from glucose metabolism were far greater than originally envisaged. The biochemical pathways employed in glucose metabolism are also numerous. The initial assumption was that glucose was oxidized using a pathway containing the *GDH* gene, resulting in the production of protons and gluconic acid (Štyriaková et al., 2012). However, the production of ethanol, acetate, CO₂ and lactate are also potential end products using pathways such as Pentose-Phosphate pathway, Entner-Doudoroff pathway and Glyoxylate shunt pathway (Fuhrer et al., 2005), that were not originally considered in the preparation of this study.

With this oversight in mind any result using the *GDH* gene assay would be non-viable as the specific pathway and end carbon product produced were not

identified in this study. Even if the amplicons isolated had matched the published *GDH* gene, this could not have been used as a proxy for weathering without identifying that gluconic acid was in fact responsible for the MPS phenotypes observed. Further physiological investigations to the MPS phenotype are required prior to this type of assay being attempted for these isolates in any future studies.

3.5. Conclusions

The culturable bacterial community identified at LMS and TCS sites do not appear to be highly specialised to their environment since the environmental conditions measured here all have deleterious effects to bacterial growth (H4). All isolates examined in the microcosm experiments were sensitive to Cu concentrations below those measured from the environment of the LMS and TCS sites and few possessed optimal growth temperatures that are experienced *in-situ*. Phosphate weathering, as measured by the MPS phenotype, appears to be a generalist function (H5).

The process of plant selection on bacterial communities, while apparent in chapter II, appears not to have enhanced the bacterial weathering capability (MPS efficacy) of the isolates examined here (H6), as there was no measurable difference in weathering efficacy between isolates from either LMS or TCS sites. This may suggest that bacterial enhanced weathering may be influenced more by abiotic factors such as temperature, substrate and liberation of heavy metals than vegetation selected bacterial diversity.

The physiological pathway used by bacteria to enhance weathering was not able to be identified in this study (H7). There is a need for more physiological

investigations in the origins of the MPS phenotype prior to any future attempts to identify this phenotype using molecular methods.

What role do Bacteria play in the weathering of critical zone soils?

4.1. Introduction

In the previous chapter and in other recent studies the potential for microbial weathering has been addressed (Abdulla, 2009, Adamo & Violante, 2000, Bennett et al., 2001, Bonneville et al., 2009, Certini et al., 2004, Coombes et al., 2011, Herrera et al., 2008, Lepleux et al., 2012, Mailloux et al., 2009, Smeaton, 2009, Štyriaková et al., 2012, Uroz et al., 2011a). However, only simple substrates such as rock or mineral phosphate have been explored in detail for the metabolic processes involved (Abdulla, 2009, Hamdali et al., 2012, Jorquera et al., 2008, Perez et al., 2007, Piervittori et al., 1994, Rodríguez et al., 2000, Santelli et al., 2009). Weathering of soil minerals by microbes has received considerable attention (Collignon et al., 2011, Eilers, 2011, Mitchell et al., 2010, Uroz et al., 2011b), but the processes and individuals responsible have yet to be conclusively determined.

The bacterial diversity and community structure at the critical zone have been investigated previously (Chapter II) and significant differences were measured, based on vegetation type inhabiting the soil surfaces. However, the weathering efficacy of isolated bacteria from this region did not differ between bacteria from TCS and LMS sites (Chapter III). Moreover, the weathering efficacy of bacteria in the previous chapter was measured using laboratory reagents and conditions; this did not explain what was likely to be observed when inoculating a more complex bacterial community to a similar weathering assay on a more intricate and natural substrate, such as soil.

Soil bacterial communities have been examined for their weathering capability on various minerals (Lepleux et al., 2012) and shown that a significant *Proteobacteria*

abundance was associated with the weathered minerals apatite and biotite. However, the effect that this taxon had on the general soil substrate was not addressed. The use of soil bacteria in studies of mineral weathering is widespread (Abdulla, 2009, Calvaruso et al., 2010, Cockell et al., 2010, Collignon et al., 2011, Lepleux et al., 2012, Mailloux et al., 2009, Štyriaková et al., 2012, Turpault et al., 2009, Uroz et al., 2011b). Yet, soils are complex substrates rich in clays, minerals and organics; the effect of bacteria on this substrate is as yet unclear.

Extensive characterisation of soil microbiology in general, as well as in this study (Chapter II), has shown that soil microbial communities are not homogeneously distributed throughout a local region (Janssen, 2006), instead particular populations are found in high densities at particular depths or in patches as a result of nutrient availability and selection pressures. Several ecologically important microbes have been recorded in high concentrations around small rock fragments within soil matrices; Certini et al. (2004) recorded the presence of several fungal and *Actinomycete* inhabitants on small rock fragments from a fine grained soil environment and concluded that the microbial communities from these rock fragments were significantly different from the surrounding soils. Both of these taxa are well studied for their role in the weathering of bedrock (Abdulla, 2009, Bååth & Anderson, 2003, Bonneville et al., 2009, Derry, 2006, Hamdali et al., 2012). Moreover, these microbial islands can potentially liberate nutrients through the weathering of insoluble minerals that are then dispersed into the wider soil communities (Uroz et al., 2011a), generating a 'nutrient hotspot'.

As discussed previously (Chapter II), nutrient availability may affect bacterial diversity and function within the sub-surface critical zone. However, the bacterial

inhabitants can interact with the soil substrate altering the nutrient and chemical signatures, further changing the substrate and subsequent bacterial communities (Sasaki et al., 1998). For example, *Shewanella* spp that are capable of Fe reduction, enhancing the weathering process (Smeaton, 2009) and liberating Fe^{2+} to the environment. In addition to Fe, the weathering of silicate substrates can liberate several minerals and elements, such as bicarbonate, silicon, copper and other materials to the water table (Dessert et al., 2003, Kump et al., 2000, Moulton & Berner, 1998). As presented in chapter III, the liberation of Cu can influence the viability of the bacterial community. This results in a co-variable reaction between bacterial and the substrate chemical composition.

The patterns described in chapter II suggest that the substrate inhabited by higher plants, and the organic species available, are key drivers of bacterial composition and structure (Bååth et al., 1995, el Zahar Haichar et al., 2008, Frostegård et al., 1991). Whereas soil microbial communities from shallow depths are generally well characterised (Janssen, 2006) and we are now beginning to understand the broad-scale patterns of soil bacterial diversity and their interactions with e.g. soil pH and land use (Grayston et al., 2004, Griffiths et al., 2011, Thomson et al., 2010). The bacterial communities of the sub-surface critical zone and their interactions with the soil matrix have not yet been properly addressed.

An attempt to correlate bacterial abundance with the chemical fingerprint of the sub-surface critical zone soils was made in chapter II. However, no correlation was made between individual taxa and chemical species. In this chapter, many of the solutes liberated during weathering were measured using a suite of chemical techniques and compared to bacterial community profiles. As no taxon was clearly

shown to have superior weathering capability for a simple substrate (Chapter III), it was thought that a more challenging substrate, such as soil, may elucidate the most efficient weathering bacteria.

4.1.1. Aims

In order to determine how the bacterial community affects the chemical solutes liberated during mineral weathering of sub-surface critical zone soils, an *in-vitro* approach was used. The aims of this chapter were to address the following hypotheses:

H8. Bacterial community structure and composition influences soil substrate.

H9. The presence of bacteria will increase solute liberation into the water table.

H10. Particular bacterial taxa are correlated with individual chemical solute liberates.

4.2. Materials and methods

4.2.1. Sample collection

Soil samples were collected according to the methods described in Chapter II from sites LMS1 and TCS1 in June 2010 (n = 18, nine samples from each site).

4.2.2. Culturing techniques and media

To determine the effect of critical zone bacterial communities on the solutes liberated from soils to the water table, a microcosm experiment was established. In order to simulate the *in-situ* conditions encountered at the critical zone, water-saturated soil microcosms were prepared in double autoclave sterilised polycarbonate flasks (FB74442, Fisher Scientific, Loughborough, UK). Soils collected from each sample site were vortexed and sieved to ≤ 2 mm to create a homogeneous substrate for each site. Sieving soils limited the effects of differences in surface-area to volume ratios on solute liberation. Flasks were prepared using 400 mL of sdH₂O and adding 200 g of air-dried soil. Twelve flasks were prepared using homogeneous soils from each sample site (TCS n = 6 and LMS n = 6).

A further 12 flasks were prepared in the same manner but containing substrate consisting of equal portions of soils from each sample site. The soils shared a broadly similar original lithology (Moulton et al., 2000), aside from differences illustrated in chapter II, i.e. higher concentrations of Si, Fe, Ca, P and Mn at TCS site and lower concentrations of Al at TCS site, when compared to the LMS site. To achieve equal composition of all samples at the start of incubations, soils from

both sample sites were pooled, air dried and sieved (≤ 2 mm), before vortexing to form a homogeneous soil matrix.

All flasks were sterilized by autoclaving at 121°C for 15 minutes; this was repeated twice to ensure sterility. Flasks were then inoculated with 20 g of biologically active soil from either LMS1 or TCS1 and incubated at 20°C for 90 days under controlled conditions. Sterilized samples without active inoculant were also prepared as controls for bacterial contamination. The sterility of these abiotic controls was tested after incubation by plating aqueous and solid phases of flask contents onto agar plates and monitoring growth. In addition, the abiotic control soils were subjected to nucleic acid extraction to determine if significant amounts of nucleic acid were present in soils either following autoclaving or as a result of contamination during incubation.

Table 4.1: A summary of the different soils used in experiment; indicating the sources of the inoculant used to biologically seed each microcosm.

Flask #	Soil Matrix	Inocula	Treatment
1 - 5	LMS soil	20 g active LMS soil	LMS _{LMS}
6	LMS soil	abiotic control	LMS _{CT}
7	TCS soil	abiotic control	TCS _{CT}
8 - 12	TCS soil	20 g active TCS soil	TCS _{TCS}
13 - 14	LMS+TCS soil	abiotic control	MIX _{CT}
15 - 19	LMS+TCS soil	20 g active TCS soil	MIX _{TCS}
20 - 24	LMS+TCS soil	20 g active LMS soil	MIX _{LMS}

The initial experimental design included abiotic controls (treatments LMS_{CT} and TCS_{CT}) to ensure that no bacterial contamination was observed during setup and incubations. However, following completion of the incubation and subsequent analysis of original soils (see Chapter II) it became evident that the soils exhibited differences in chemical composition even though they shared broadly similar

original lithologies. As a result of this dissimilarity, all solute analysis results from treatments LMS_{LMS} and TCS_{TCS} had to be adjusted by subtracting their measured control values from measured liberates to give an approximate measure of bacterially-induced solute liberation (Results; Table 4.3); however as only one control was prepared for each treatment, the confidence in this approach was low. This issue was addressed and accounted for in treatments MIX_{TCS} and MIX_{LMS}, as treatment soils were equal at the initiation of experiment. The measured values could therefore be analysed without correction (Results; Table 4.2).

4.2.3. Separation of soils from solution.

Chemical analysis was conducted on the aqueous phase of the soil slurry to detect solutes liberated from the soil to the water. To achieve this the solid and aqueous phases were separated by filtering the samples into acid-washed glass containers using 0.45 µm polycarbonate filters (Whatman, Maidstone, Kent). The filtrate was then aliquotted into separate vials for solute analysis whereas the remaining solid phase was stored at -20°C for subsequent microbial analyses.

4.2.4. Extraction of nucleic acids.

Representative samples of nucleic acids were required to determine the microbial community composition of the soils. Extraction of total nucleic acids from soil was conducted on the solid phase from all 24 microcosm incubations according to the method described by Griffiths et al. (2000), with minor modifications to enhance nucleic acid yield; these consisted of a second bead-beating step, with a one-minute submersion of lysis tubes in an ice bath to preserve any nucleic acids between steps; and the addition of 1 µL of GlycoBlue (Invitrogen, Paisley, UK) to each extract during precipitation of nucleic acids to enhance the visibility of the

nucleic acids. The presence of nucleic acids in the final extracts was confirmed by 1% agarose gel electrophoresis with ethidium bromide staining.

4.2.5. Solute liberation analysis

All chemical analysis and quantification of solutes present in the aqueous phase was conducted by the aquatic chemistry laboratories at the Centre for Ecology and Hydrology (Wallingford, UK). Briefly, measurements of pH were conducted using an analytical pH meter (PHM210; Radiometer Analytical, Lyon, France). Gran alkalinity was determined as per (Neal & Hill, 1994), using acidimetric titration over the pH range 3.0 - 4.0 to reflect the bicarbonate buffering capacity of the samples.

Conductivity and total dissolved solids (TDS) were measured using a multi-parameter Ultrameter II (Myron L, CA, USA).

Soluble reactive phosphorus (SRP) and ammonium (NH_4) were measured simultaneously using a colourimeter (AA3; SEAL Analytic LTD, Fareham, UK). The SRP was determined using the molybdenum blue method described by (Holman, 1943), and NH_4 by the salicylate method as described by (Kempers & Zweers, 1986). A six-point calibration was used for both, with a range of 0-1.5 $\text{mg PO}_4 \text{ L}^{-1}$ and 0-0.2 $\text{mg NH}_4 \text{ L}^{-1}$. Concentrations of silicon (Si) were measured using a colourimeter (AA3; SEAL Analytic LTD, Fareham, UK) using the molybdenum blue method (Holman, 1943). A six-point calibration was used, with a range of 0-10.0 mg Si L^{-1} . Fluorine (F), Chlorine (Cl), Nitrogen Dioxide (NO_2), Nitrate (NO_3) and Sulphate (SO_4) were analysed simultaneously using ion chromatography (Dionex AS50; Thermo Scientific, Essex, UK), with a sodium bicarbonate/sodium carbonate eluent mix. A six-point calibration was used for each nutrient, with

ranges as follows: 0 - 2.5 F mg L⁻¹; 0 - 50 mg Cl L⁻¹; 0 - 5.0 mg NO₂ L⁻¹; 0 - 50 mg NO₃ L⁻¹; 0 - 150 mg SO₄ L⁻¹.

Sodium (Na), Potassium (K), Calcium (Ca), Magnesium (Mg), Iron (Fe), Manganese (Mn), Copper (Cu), and Aluminium (Al) were analysed simultaneously using an inductively coupled plasma-optical emission spectrophotometer (ICP-OES) (Optima 2100 DV; Perkin Elmer, Ma, USA). Samples and calibration solutions were acidified to 1% vol/vol using concentrated nitric acid. A five-point calibration was used for Na, K, and Mg; and a four-point calibration for Ca, Fe, Mn, Cu, Al. Ranges as follows: 0 - 100 mg Na L⁻¹; 0 - 25 mg K L⁻¹; 0 - 225 mg Ca L⁻¹; 0 - 25 mg Mg L⁻¹; 0 - 5000 µg Fe L⁻¹; 0 - 5000 µg Mn L⁻¹; 0 - 5000 Cu µg L⁻¹; 0 - 5000 Al µg L⁻¹.

4.2.6. tRFLP analysis

To determine the bacteria community composition, tRFLP analysis was performed on extractions from inoculated microcosms (n = 20) following the method outlined in Chapter II.

4.2.7. 454 high throughput NG sequencing

The use of 454 sequencing permitted the taxonomic identification of the tRFs. Although the 16S rRNA sequence database established in Chapter II was extensive, there were unique peaks observed in the tRFLP electropherograms, here that could not be identified and an appendix to the sequence database was required.

Amplification and analysis using 454 high-throughput NG sequencing was conducted on two samples (# 2 and 8, Table 4.1.) using the methods outlined in Chapter II. The use of only two samples did not permit a robust comparison of the

bacterial community between microcosms; however it was sufficient to permit taxonomic identification of the more representative tRF fragments. Subsequent barcode tag removal, primer removal and analysis of all sequences was conducted using MOTHUR software (Schloss et al., 2009). Sequences were filtered to ensure that all sequences were free of ambiguous bases and at least 300 n.t. in length. Chimeric sequences were removed using ChimeraSlayer (Haas et al., 2011) in QIIME (Caporaso et al., 2010). Phylogenetic tree generation was conducted using the FastTree (v2) package in QIIME (Price et al., 2009) by converting all sequences to OTUs (97% similarity to representative sequences). All sequences were aligned against the Silva sequence database (Pruesse et al., 2007) and taxonomy generated using the Naïve Bayesian rRNA Classifier v1.0 analysis tool within the RDP classifier (Wang et al., 2007b).

In-silico digestion of all sequences was conducted using TRFLPMAP software (http://nebc.nerc.ac.uk/cgi-bin/trflp0_2.cgi). An approximate taxon was assigned to each tRF using the criteria outlined in Chapter II.

4.2.8. Statistical analysis

Bacterial community data (tRF profiles), were compared between treatments using analysis of similarity (ANOSIM). The cumulative effect of individual tRFs on the overall differences between treatments was assessed by SIMPER analysis. The dissimilarity of bacterial communities was assessed in ordinations; all treatments were visualised together using a non-metric multidimensional scaling (NMDS) ordination; This was selected due to the large number of zero values present in data matrix as the use of NMDS limited the 'Horseshoe effect' (Kendall, 1971). Treatments LMS_{LMS} and TCS_{TCS} were plotted separately from treatments MIX_{TCS} and MIX_{LMS} using canonical correspondence analysis (CCA). All ordinations used

Bray-Curtis dissimilarity measures, with the exception of the control-corrected solute liberates, which included negative values, therefore the Manhattan index was employed. Vectors of solute liberation data were determined using EnvFit function in the vegan Package in R version 2.15.1 (Oksanen et al., 2012). Individual solute concentrations were compared between treatments using Mann Whitney U tests.

All data obtained from tRF methods were converted to Shannon-Weiner (Shannon-weaver) diversity indices (H'). As discussed in Chapter II, diversity indices on tRFLP data has an increased error rendering the results unsuitable for comparison in the wider scientific literature; however their use here as a simple comparison was acceptable (Blackwood et al., 2007), as errors were accounted for when examining the results. For comparisons of diversity indices a Kruskal-Wallace test of difference was used to compare all treatments. Whereas, diversity indices comparison between treatments LMS_{LMS} and TCS_{TCS} and between MIX_{LMS} and MIX_{TCS} were conducted using a Mann Whitney U test of difference.

4.3. Results

4.3.1. Solute liberation analysis

Analysis of the solutes in the aqueous phase of treatments LMS_{LMS} and TCS_{TCS} showed that the pH and F concentration were generally similar between treatments (Table 4.2). Treatment LMS_{LMS} had higher concentrations of Si, NO_2 , NO_3 , SO_4 and Cu. Whereas, alkalinity, conductivity, TDS, SRP, NH_4 , Cl, Na, K, Ca, Mg, Fe and Mn were all lower than in treatment TCS_{TCS} . The solutes in the aqueous phase of treatments MIX_{TCS} and MIX_{LMS} only showed differences in F and Al concentrations between the two treatments but the differences were not statistically significant (Mann Whitney U; F, $p = 0.31$; Al, $p = 0.055$).

As the soils used in treatments LMS_{LMS} and TCS_{TCS} differed from those used in MIX_{TCS} and MIX_{LMS} , no direct comparison of the raw data was made between the two experiments; however control-corrected measures (Table 4.3) provided a notional comparative measure of microbially-induced solute liberation from the solid to the aqueous phase. The biotic solute liberation significantly differed among the four treatments' (ANOSIM, $R = 0.4723$, $p < 0.001$, distance = Manhattan). Comparisons of the control-corrected solute liberation between treatments LMS_{LMS} and TCS_{TCS} therefore indicated that there was a difference in the chemical composition of the aqueous phase of treatments (ANOSIM, $R = 0.636$ $p < 0.01$, distance = Manhattan).

As treatments MIX_{TCS} and MIX_{LMS} had the same initial substrate, it was possible to test the total solute liberates directly; however, no statistically significant difference was observed (ANOSIM, $R = -0.06$, $p = 0.524$). Comparison of solute liberation from treatments MIX_{TCS} and MIX_{LMS} with abiotic solute liberation from treatment

MIX_{CT} showed there were significant differences of the chemical fingerprints (ANOSIM, $R = 0.3418$, $p = 0.021$); this indicated that while the solute liberates composition was the same between biotic treatments despite using different bacterial inocula, there were differences between biotic and abiotic solute liberation overall.

Table 4.2: Mean composition of solute liberates from soil to aqueous phase of flasks for each treatment. Means and standard deviations given are raw data. Control values are present at base of table.

Treatment		pH	Alk	Conductivity	TDS	SRP	NH ₄	Si	F	Cl	NO ₂
			$\mu\text{equ L}^{-1}$	$\mu\text{S cm}^{-1}$	ppm	$\mu\text{g L}^{-1}$	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}
LMS _{LMS}	Mean	6.88	385.00	95.42	60.18	3.60	0.12	9.84	0.08	2.91	0.75
	SD	0.09	29.01	1.10	0.70	2.51	0.04	0.14	0.12	0.08	1.04
TCS _{TCS}	Mean	6.80	1908.20	249.74	161.54	6.20	1.28	8.36	0.08	6.66	0.03
	SD	0.06	629.52	56.11	37.72	3.11	0.23	0.41	0.04	0.36	0.06
MIX _{LMS}	Mean	7.03	287.80	107.27	72.50	4.40	0.18	10.98	0.04	1.20	0.28
	SD	0.08	20.09	13.66	11.65	2.51	0.34	0.22	0.02	0.10	0.24
MIX _{TCS}	Mean	6.97	316.00	116.84	77.05	3.40	0.09	11.04	0.02	1.41	5.07
	SD	0.26	87.22	6.02	3.94	1.52	0.11	0.28	0.01	0.26	6.74
LMS _{CT}	Actual	6.79	416.00	79.96	50.46	8.00	0.21	9.65	0.09	2.99	0.09
TCS _{CT}	Actual	6.97	935.00	159.90	101.30	22.00	1.39	9.62	0.07	6.37	0.00
MIX _{CT}	Mean	7.08	215.00	100.89	66.40	10.50	0.43	10.72	0.05	1.33	0.15
	SD	0.08	50.91	14.44	9.57	6.36	0.55	0.76	0.05	0.35	0.16
Treatment		NO ₃	SO ₄	Na	K	Ca	Mg	Fe	Mn	Cu	Al
		mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1}	$\mu\text{g L}^{-1}$	$\mu\text{g L}^{-1}$	$\mu\text{g L}^{-1}$	$\mu\text{g L}^{-1}$
LMS _{LMS}	Mean	3.18	11.94	11.92	0.59	3.20	1.14	179.56	300.10	251.22	10.66
	SD	1.12	0.48	0.34	0.02	0.18	0.06	81.88	47.52	89.27	3.27
TCS _{TCS}	Mean	0.51	0.27	20.39	1.55	13.95	9.28	14211.70	4141.61	62.02	25.64
	SD	0.35	0.22	1.68	0.14	3.45	2.57	7979.63	1224.25	32.95	9.18
MIX _{LMS}	Mean	17.48	11.17	16.87	2.46	11.95	2.06	52.85	668.75	25.54	45.00
	SD	11.14	0.92	2.70	1.02	1.54	0.40	8.26	261.64	5.39	10.29
MIX _{TCS}	Mean	15.16	11.98	16.21	2.03	10.91	2.20	52.89	756.94	23.44	34.71
	SD	8.85	1.09	1.56	0.48	2.64	0.36	7.99	302.10	3.11	8.38
LMS _{CT}	Actual	0.22	7.72	11.93	0.63	2.33	0.77	117.19	133.00	295.57	13.51
TCS _{CT}	Actual	0.55	1.36	17.27	1.22	7.58	4.07	12413.65	1736.78	318.97	40.78
MIX _{CT}	Mean	11.66	11.57	14.51	1.95	11.18	1.81	488.85	518.25	37.88	504.69
	SD	16.32	0.06	1.15	0.45	1.69	0.43	590.64	73.72	13.61	659.46

Table 4.3: Mean biotic solute liberation of treatments LMS_{LMS} and TCS_{TCS}. All recorded values have had control values deducted. Negative values indicate records lower than abiotic control and positive values indicate records higher than abiotic control.

<i>Treatment</i>		<i>pH</i>	<i>Alk</i> <i>μequ L⁻¹</i>	<i>Conductivity</i> <i>μS cm⁻¹</i>	<i>TDS</i> <i>ppm</i>	<i>SRP</i> <i>μg L⁻¹</i>
LMS _{LMS}	mean	0.094	-31.000	15.456	9.718	-4.400
	SD	0.092	29.009	1.102	0.698	2.510
TCS _{TCS}	mean	-0.170	973.200	89.840	60.240	-15.800
	SD	0.059	629.515	56.111	37.719	3.114
<i>Treatment</i>		<i>NO₃</i> <i>mg L⁻¹</i>	<i>SO₄</i> <i>mg L⁻¹</i>	<i>Na</i> <i>mg L⁻¹</i>	<i>K</i> <i>mg L⁻¹</i>	<i>Ca</i> <i>mg L⁻¹</i>
LMS _{LMS}	mean	2.962	4.215	-0.004	-0.034	0.870
	SD	1.121	0.476	0.340	0.016	0.182
TCS _{TCS}	mean	-0.043	-1.099	3.114	0.325	6.373
	SD	0.354	0.224	1.679	0.143	3.451

<i>Treatment</i>		<i>NH₄</i> <i>mg L⁻¹</i>	<i>Si</i> <i>mg L⁻¹</i>	<i>F</i> <i>mg L⁻¹</i>	<i>Cl</i> <i>mg L⁻¹</i>	<i>NO₂</i> <i>mg L⁻¹</i>
LMS _{LMS}	mean	-0.094	0.190	-0.004	-0.081	0.660
	SD	0.044	0.137	0.118	0.075	1.044
TCS _{TCS}	mean	-0.112	-1.262	0.013	0.290	0.029
	SD	0.230	0.408	0.040	0.360	0.064
<i>Treatment</i>		<i>Mg</i> <i>mg L⁻¹</i>	<i>Fe</i> <i>μg L⁻¹</i>	<i>Mn</i> <i>μg L⁻¹</i>	<i>Cu</i> <i>μg L⁻¹</i>	<i>Al</i> <i>μg L⁻¹</i>
LMS _{LMS}	mean	0.373	62.373	167.104	-44.348	-2.854
	SD	0.056	81.878	47.524	89.272	3.273
TCS _{TCS}	mean	5.200	1798.057	2404.822	-256.959	-15.138
	SD	2.574	7979.630	1224.251	32.947	9.178

Statistical comparisons of individual solutes between treatments LMS_{LMS} and TCS_{TCS} or MIX_{TCS} and MIX_{LMS} were made (Table 4.4). No significant differences in any of the solute concentrations between treatments MIX_{TCS} and MIX_{LMS} were apparent. Comparisons between the control-adjusted values of treatments LMS_{LMS}

and TCS_{TCS} indicated that five of the 20 solutes measured were significantly different i.e. pH, Si, NO₃, SO₄ and Cu.

Comparisons of the raw data showed that the mean concentrations of Fe were 80 times higher in treatment TCS_{TCS} than LMS_{LMS}. Examination of the control-corrected means showed that biotic release of Fe was c. 27 times higher in treatment TCS_{TCS} (Table 4.2); however, variability among samples was so high, that this difference was not significant.

Table 4.4: Results of Mann Whitney U tests of difference between the two sets of treatments. All significant results are indicated in bold and test statistic (U) provided.

<i>Treatments → Elements ↓</i>	<i>LMS_{LMS} and TCS_{TCS}</i>		<i>MIX_{TCS} and MIX_{LMS}</i>	
	U	<i>p</i>	U	<i>p</i>
pH	32	0.025	8	0.420
Alkalinity	7	0.093	15	0.690
Conductivity	13	0.484	18	0.309
TDS	13	0.484	16	0.547
SRP	30	0.064	10	0.653
NH ₄	16	0.81	18	0.263
Si	31	0.044	16	0.529
F	12	0.393	22	0.055
Cl	12	0.393	6	0.222
NO ₂	27	0.158	8	0.420
NO₃	34	0.008	15	0.690
SO₄	36	0.002	7	0.309
Na	24	0.393	9	0.547
K	11	0.309	15	0.690
Ca	7	0.093	16	0.547
Mg	6	0.064	9	0.547
Fe	18	1	12	1
Mn	6	0.064	10	0.690
Cu	31	0.041	13	1
Al	29	0.093	20	0.150

4.3.2. tRFLP analysis

4.3.2.1. Site-specific soils

Statistical analysis and ordination of the bacterial community fingerprints measured using tRFLP analysis revealed that the bacterial communities from treatments LMS_{LMS} and TCS_{TCS} were highly dissimilar (ANOSIM, $R = 1$, $p < 0.01$), and were separated along the primary CCA axis (Figure 4.1). Particular solutes, such as SI, Cu, SO₄, NO₃ and pH were all higher in concentration in treatment LMS_{LMS}, whereas solutes Na, K, Mg and DOC were all higher in treatment TCS_{TCS}. These solutes were correlated with the different treatments within the ordination. Noteworthy from this was the significance of DOC as this can be a significant driver of microbial diversity in soils (Grayston et al., 1998).

Taxonomic identification and SIMPER analysis of the tRFs observed in treatments LMS_{LMS} and TCS_{TCS} highlighted the dominance of *Clostridia*, specifically *Negativicutes* (Table 4.5), represented by the tRF at 255 n.t.. The closest taxonomic match to this tRF was the genus *Sporotalea* of the family *Veillonellaceae*.

The second most abundant tRF (129 n.t.) in treatment TCS_{TCS} was *Clostridia* also a member of the *Firmicutes*, however the closest match for this tRF was the genus *Desulfitobacterium* of the family *Peptococcaceae*. The class *Clostridia* was identified for a further 12 tRFs, however their low abundance limited the depth of taxonomic identification. Their individual contributions to bacterial community

differences was low, yet combined, the remainder of *Clostridia* explained 31.48% of the difference in bacterial community composition between treatments.

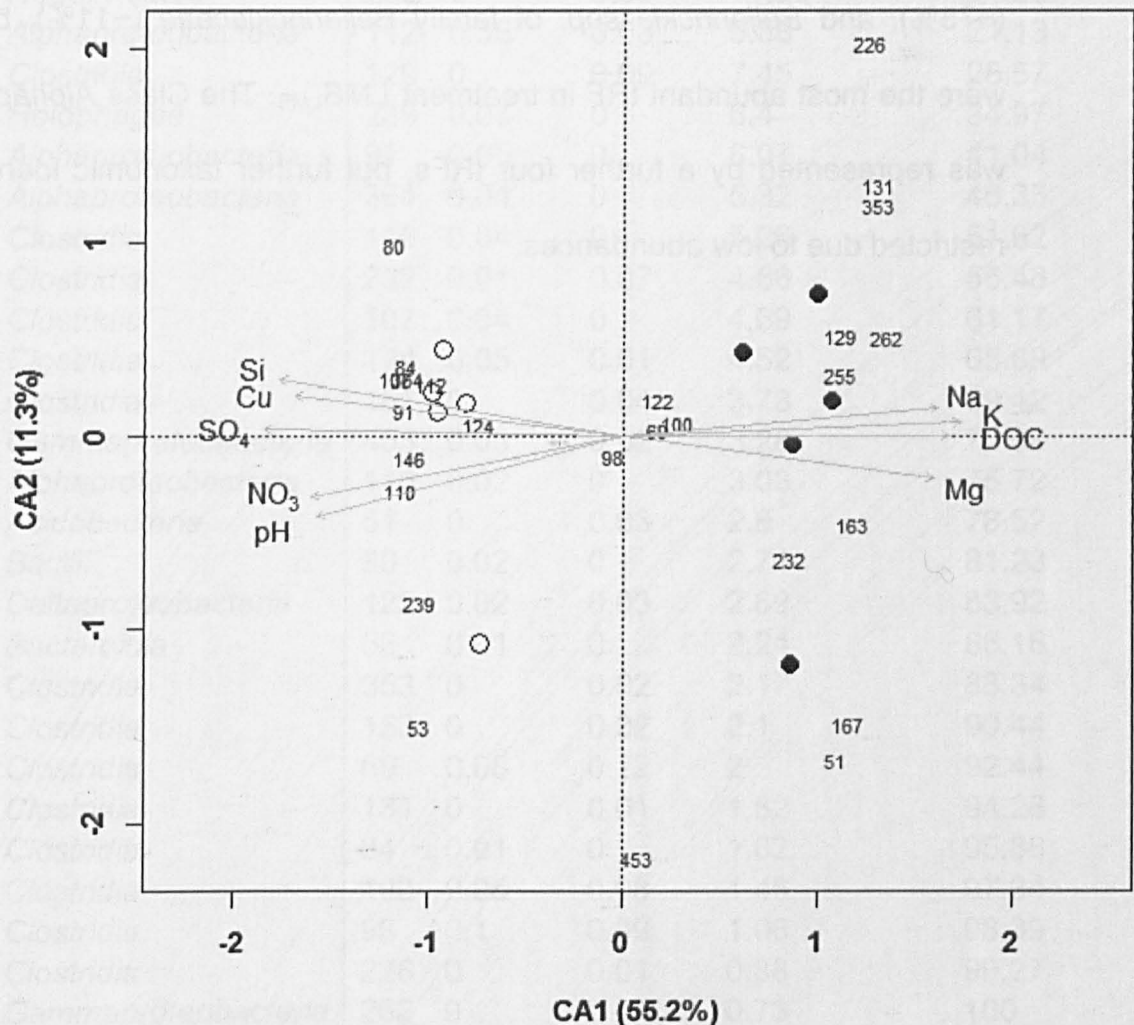


Figure 4.1: Biplot of bacterial communities from treatments LMS_{LMS} (●) and TCS_{TCS} (○). Environmental variables are denoted by gray arrows, where the length and direction of the arrow indicates the strength of their correlation with bacterial taxa all environmental variables shown were significantly correlated to bacterial community composition at $p \leq 0.01$.

The differences in *Alphaproteobacteria* also had a substantial influence on bacterial community. Almost 10% of the variance among communities from each treatment was attributed to *Alphaproteobacteria*, identified by the tRF 112 n.t.. The tRF was predominantly represented by *Afipia* spp. of family *Bradyrhizobiaceae* (~75%), and *Beijerinckia* spp. of family *Beijerinckiaceae* (~11%), both of which were the most abundant tRF in treatment LMS_{LMS}. The Class *Alphaproteobacteria* was represented by a further four tRFs, but further taxonomic identification was restricted due to low abundances.

Table 4.5: SIMPER analysis of differences in bacterial communities in treatments LMS_{LMS} and TCS_{TCS}. Comparisons are based on the relative abundances of each tRF; the individual and cumulative relative contributions to overall dissimilarity are given for each tRF.

Taxa (Class)	<i>Treatment</i>			Individual contribution %	Cumulative %
	tRF	LMS _{LMS}	TCS _{TCS}		
<i>Negativicutes</i>	255	0	0.35	11.25	11.25
<i>Alphaproteobacteria</i>	112	0.35	0.03	9.88	21.13
<i>Clostridia</i>	129	0	0.09	7.45	28.57
<i>Holophagae</i>	239	0.07	0	6.4	34.97
<i>Alphaproteobacteria</i>	91	0.05	0	6.07	41.04
<i>Alphaproteobacteria</i>	364	0.04	0	5.32	46.36
<i>Clostridia</i>	146	0.04	0	5.26	51.62
<i>Clostridia</i>	232	0.01	0.07	4.86	56.48
<i>Clostridia</i>	107	0.04	0	4.69	61.17
<i>Clostridia</i>	124	0.05	0.01	4.52	65.69
<i>Clostridia</i>	167	0	0.04	3.73	69.42
<i>Gammaproteobacteria</i>	453	0.03	0.02	3.28	72.69
<i>Alphaproteobacteria</i>	110	0.02	0	3.03	75.72
<i>Acidobacteria</i>	51	0	0.03	2.8	78.52
<i>Bacilli</i>	80	0.02	0	2.71	81.23
<i>Deltaproteobacteria</i>	122	0.02	0.03	2.69	83.92
<i>Bacteroidia</i>	53	0.01	0	2.24	86.16
<i>Clostridia</i>	353	0	0.02	2.17	88.34
<i>Clostridia</i>	163	0	0.02	2.1	90.44
<i>Clostridia</i>	69	0.08	0.12	2	92.44
<i>Clostridia</i>	131	0	0.01	1.82	94.26
<i>Clostridia</i>	84	0.01	0	1.62	95.88
<i>Clostridia</i>	100	0.05	0.08	1.46	97.34
<i>Clostridia</i>	98	0.1	0.09	1.06	98.39
<i>Clostridia</i>	226	0	0.01	0.88	99.27
<i>Gammaproteobacteria</i>	262	0	0	0.73	100

The Class *Holophagae* accounted for 6.4% of the differences in the bacterial communities between treatments LMS_{LMS} and TCS_{TCS} (tRF 239 n.t.). This *Acidobacteria* was the most abundant of the Phyla in this study. It is a well-studied *Acidobacteria* and therefore taxonomic identification was possible down to genus

level with confidence. This tRF consisted exclusively of species of the genus *Geothrix* of the family *Holophagaceae*.

4.3.2.2. Composite soils

There was no relationship between tRF relative abundances and ordination space in the composite soil treatments (MIX_{LMS} and MIX_{TCS}). Although treatments appeared to cluster discreetly the tRFs were randomly distributed (Figure 4.2); the samples from treatment MIX_{LMS} were much more variable and there were no clear differences between the two treatments (ANOSIM, $R = 0.344$, $p = 0.056$). There was no evidence of any relationships between solute liberates (Table 4.3) and bacterial community composition.

SIMPER analysis showed that *Alphaproteobacteria* were highly influential in determining bacterial composition (Table 4.6). *Clostridia*, represented by the genus *Desulfitibacter* (tRF 107 n.t.), had the highest individual influence on bacterial community composition. However the number of tRFs identified as *Clostridia* were lower than observed in treatments LMS_{LMS} and TCS_{TCS}. The most abundant bacterial class in treatments MIX_{TCS} and MIX_{LMS} was the *Alphaproteobacteria*, represented by the genus *Caulobacter* in several tRFs (109, 110 and 111 n.t.).

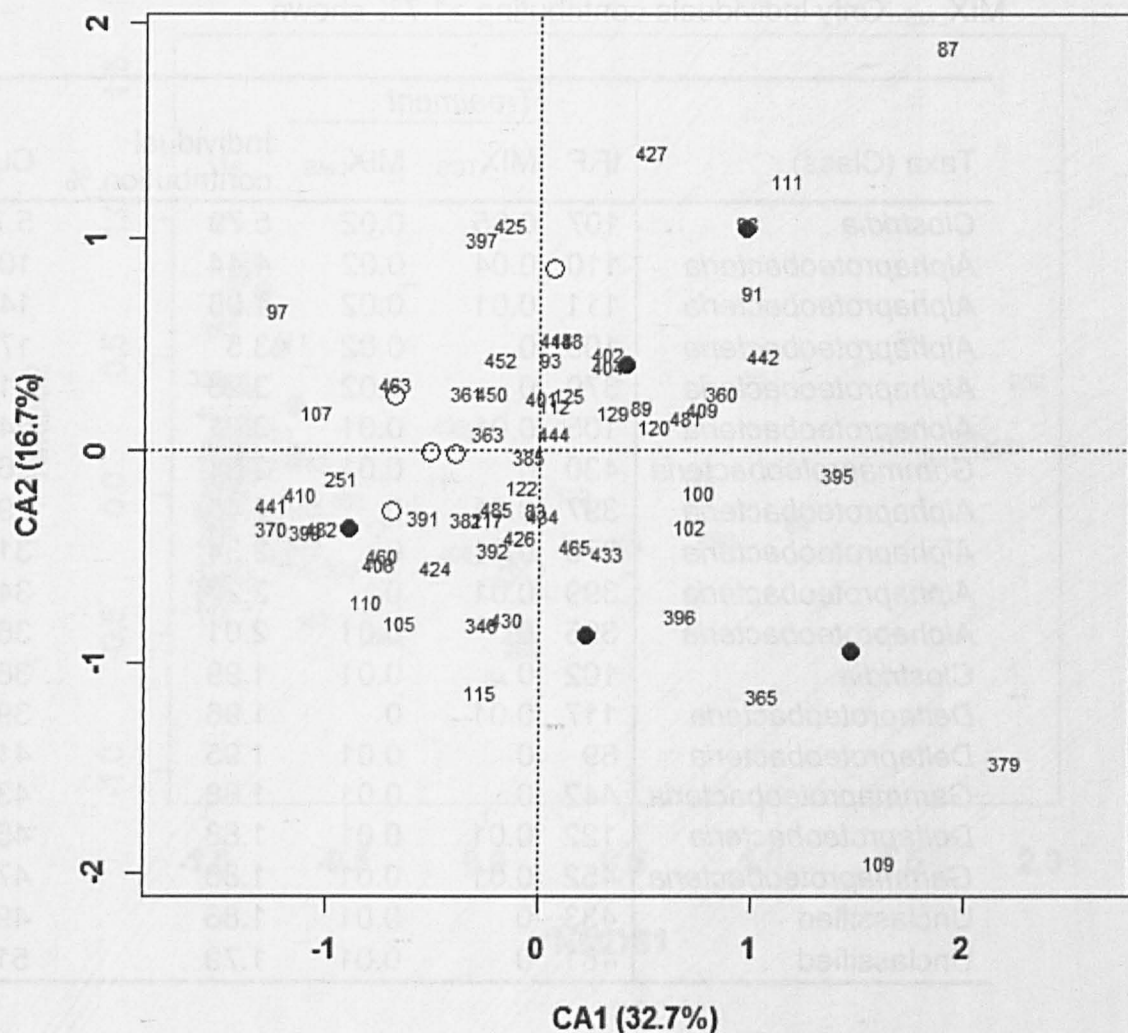


Figure 4.2: Ordination biplot of the bacterial communities from treatments MIX_{TCS} (○) and MIX_{LMS} (●). Numbers given are individual tRFs.

Table 4.6: SIMPER analysis indicating the relative contribution of tRFs to differences in bacterial community structure between treatments MIX_{TCS} and MIX_{LMS}. Only individuals contributing >1.7% shown.

Taxa (Class)	Treatment			Individual contribution %	Cumulative %
	tRF	MIX _{TCS}	MIX _{LMS}		
<i>Clostridia</i>	107	0.05	0.02	5.79	5.79
<i>Alphaproteobacteria</i>	110	0.04	0.02	4.44	10.23
<i>Alphaproteobacteria</i>	111	0.01	0.02	4.08	14.31
<i>Alphaproteobacteria</i>	109	0	0.02	3.5	17.81
<i>Alphaproteobacteria</i>	379	0	0.02	3.28	21.09
<i>Alphaproteobacteria</i>	105	0.01	0.01	3.01	24.1
<i>Gammaproteobacteria</i>	430	0	0.01	2.81	26.91
<i>Alphaproteobacteria</i>	397	0.01	0	2.48	29.39
<i>Alphaproteobacteria</i>	370	0.01	0	2.34	31.73
<i>Alphaproteobacteria</i>	399	0.01	0	2.27	34
<i>Alphaproteobacteria</i>	395	0	0.01	2.01	36.01
<i>Clostridia</i>	102	0	0.01	1.99	38
<i>Deltaproteobacteria</i>	117	0.01	0	1.96	39.95
<i>Deltaproteobacteria</i>	89	0	0.01	1.95	41.9
<i>Gammaproteobacteria</i>	442	0	0.01	1.88	43.78
<i>Deltaproteobacteria</i>	122	0.01	0.01	1.88	45.67
<i>Gammaproteobacteria</i>	452	0.01	0.01	1.86	47.53
Unclassified	433	0	0.01	1.86	49.38
Unclassified	481	0	0.01	1.79	51.17

4.3.2.3. All treatments

When all treatments were plotted together using NMDS, the samples exhibited similar groupings as presented above (Figure 4.3). There was a significant overall difference among the treatments (ANOSIM, $R = 0.9763$, $p \leq 0.001$); however this was likely due to the dominance of several novel tRFs in treatment TCS_{TCS}.

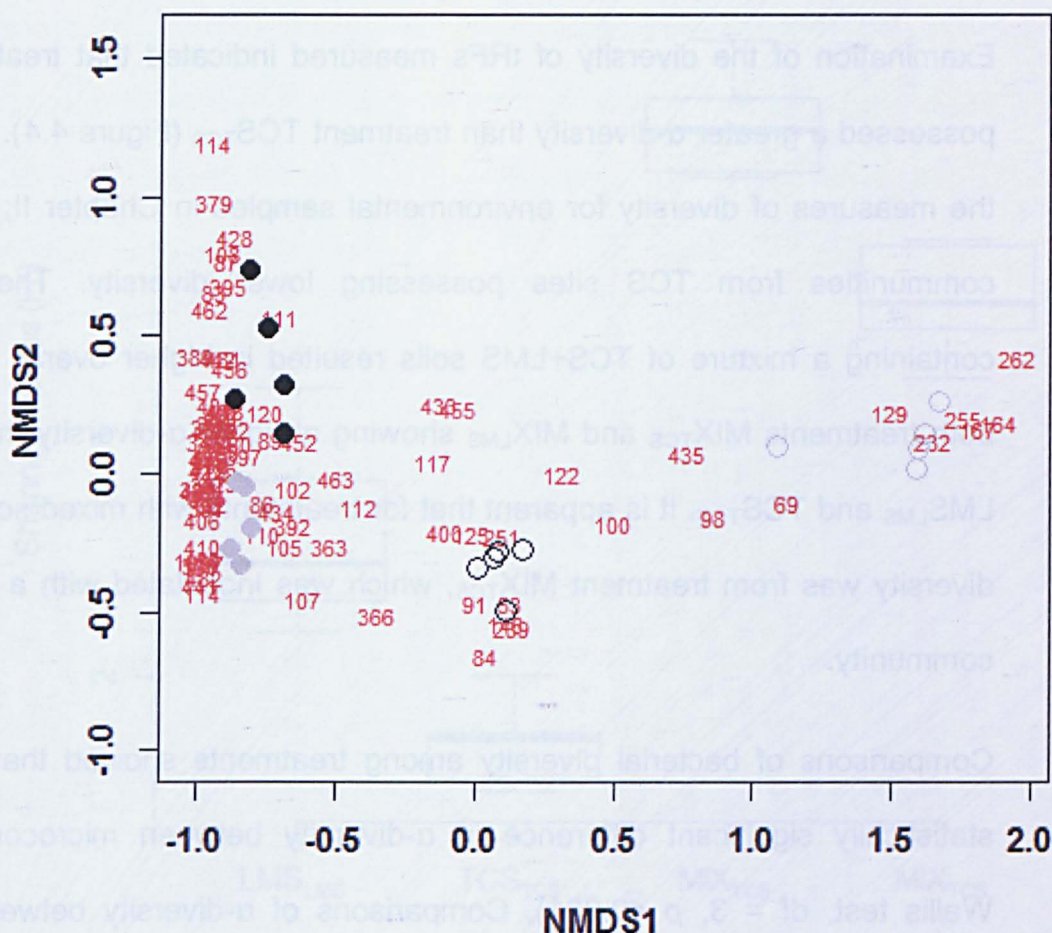


Figure 4.3: NMDS ordination showing the tRF dissimilarity of all microcosm treatments. Evident is the difference in bacterial community for treatment LMS_{LMS} (○) and TCS_{TCS} (○) and the resulting convergence of treatment MIX_{LMS} (●) and MIX_{TCS} (●) bacterial communities. Ordination stress = 0.065.

The high number of tRFs observed in Figure 4.3 clustering around Treatments MIX_{TCS} and MIX_{LMS} indicates the similarity between the two. The separation between treatments LMS_{LMS} and TCS_{TCS} shows the tRFs aligned with samples;

these were clearly observed in Table 4.5 as important tRFs for bacterial community (tRFs: i.e. 255, 164 and 91 n.t.).

Examination of the diversity of tRFs measured indicated that treatment LMS_{LMS} possessed a greater α -diversity than treatment TCS_{TCS} (Figure 4.4). This matched the measures of diversity for environmental samples in Chapter II; with bacterial communities from TCS sites possessing lower diversity. The microcosms containing a mixture of TCS+LMS soils resulted in higher overall diversity, with both treatments MIX_{TCS} and MIX_{LMS} showing a higher α -diversity than treatments LMS_{LMS} and TCS_{TCS}. It is apparent that for treatments with mixed soils the greater diversity was from treatment MIX_{TCS}, which was inoculated with a TCS bacterial community.

Comparisons of bacterial diversity among treatments showed that there was a statistically significant difference in α -diversity between microcosms (Kruskal-Wallis test, $df = 3$, $p < 0.001$). Comparisons of α -diversity between treatments LMS_{LMS} and TCS_{TCS} (Mann Whitney U, $U = 15$, $p = 0.012$) as well as between MIX_{TCS} and MIX_{LMS} (Mann Whitney U, $U = 40$, $p = 0.012$) showed that the diversities were not equal.

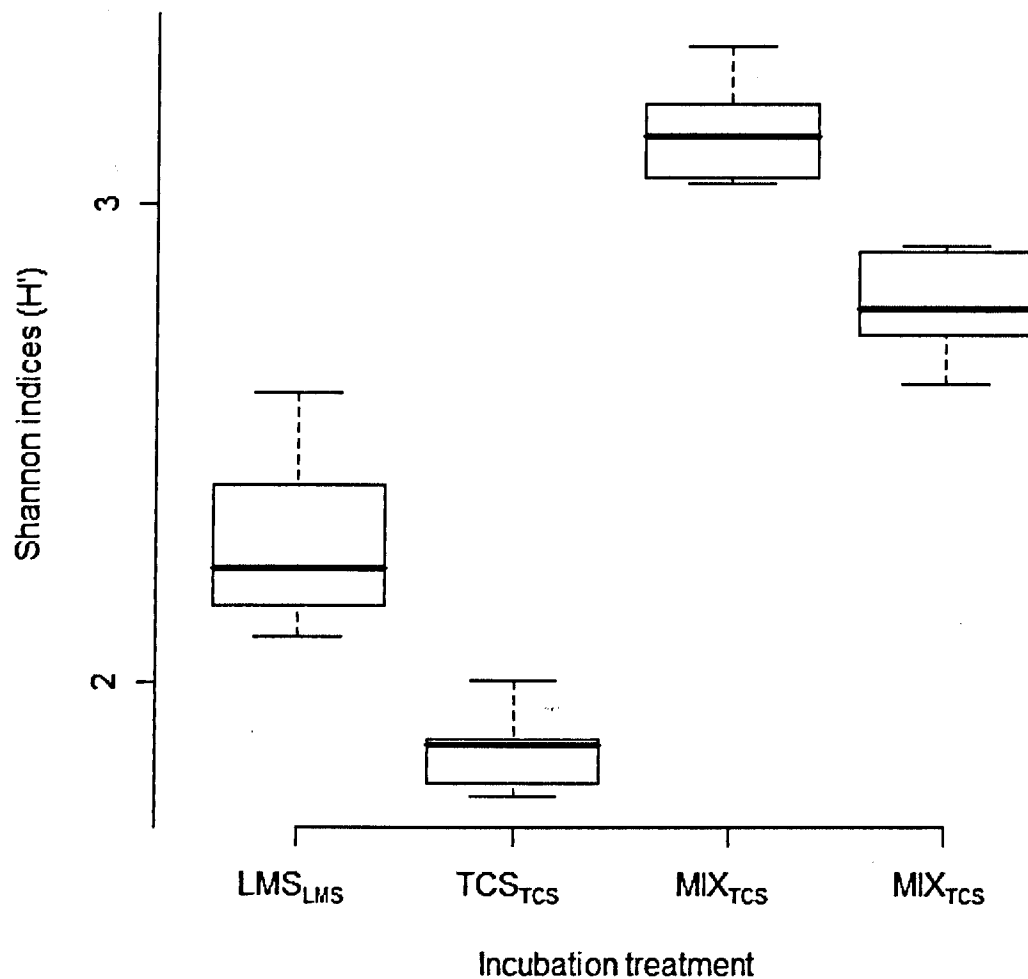


Figure 4.4: Box-whisker plot showing the α -diversity (H') for each of the four treatments. Whiskers indicate 95% CI.

4.3.3. 454 high throughput NG sequencing

454 NG pyrosequencing was carried out primarily to provide taxonomic identification of tRF data and also examine the bacterial communities at a greater resolution. Following removal of sub-quality sequences, treatment LMS_{LMS} had

2170 partial 16S rRNA gene sequences and treatment TCS_{TCS} had 1871. Sequence analysis from treatments LMS_{LMS} and TCS_{TCS} identified 169 OTUs and 84 OTUs, respectively (Figure 4.5), corroborating the findings obtained through tRFLP analysis, with greater species richness in samples inoculated with LMS soil.

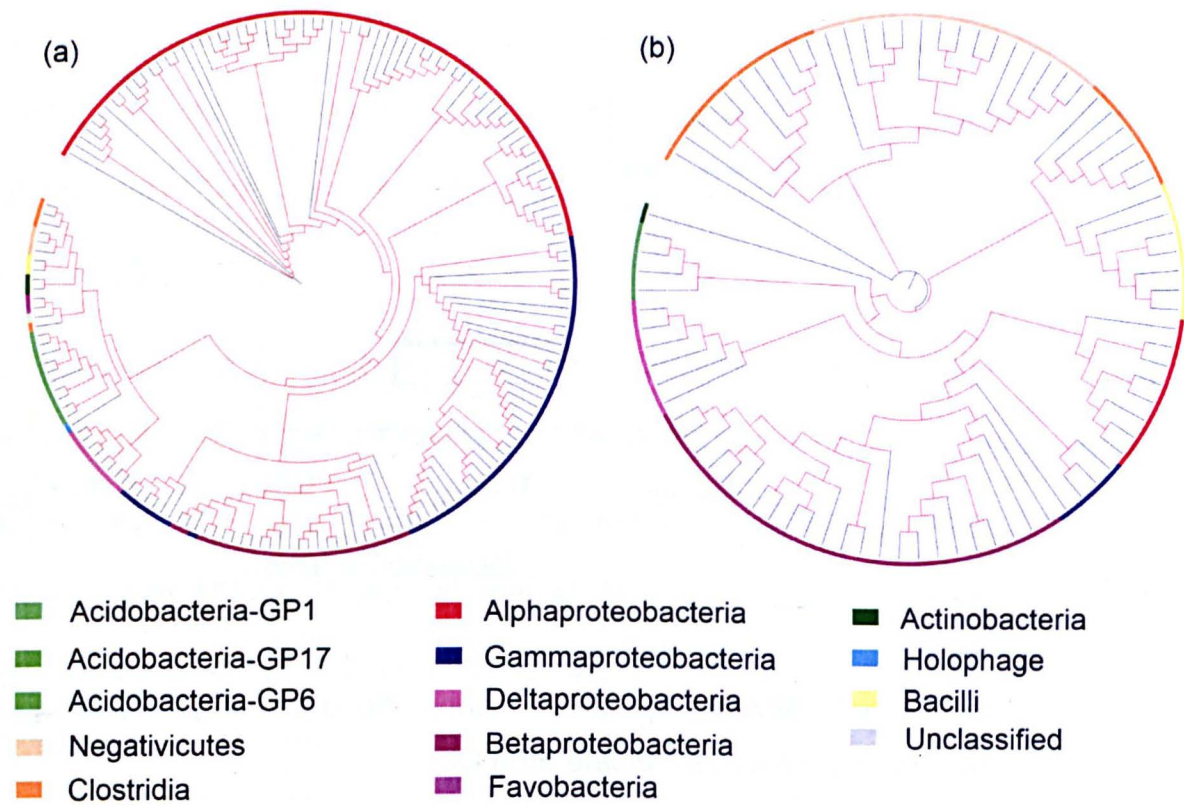


Figure 4.5: (a) Treatment LMS_{LMS} (b) Treatment TCS_{TCS}. OTUs (97% similarity between sequences) were plotted using an approximate maximum likelihood phylogenetic tree. All branches highlighted in red indicate a bootstrap value >0.5. tree visualized using ITOL (Letunic & Bork, 2011).

The dominant phyla observed in treatment LMS_{LMS} were *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. The *Alphaproteobacteria* group consisted of 62% *Rhizobiales*, 16% *Sphingomonadales* and 11% *Caulobacteriales*. A notable proportion of *Gammaproteobacteria* were *Pseudomonadales* (c. 66%). All OTUs identified as *Betaproteobacteria* were from the order *Burkholderiales*, family *Oxalobacteraceae*.

Firmicutes and *Betaproteobacteria* each accounted for c. 29% of the OTUs in treatment TCS_{TCS}. Similarly to treatment LMS_{LMS}, all members of the *Betaproteobacteria* belonged to the family *Oxalobacteraceae*. Within the *Firmicutes*, most OTUs were representatives of the family *Veillonellaceae*. However, the data did not permit identification to genus level. The abundance of *Holophagae* in the tRF data was not apparent in the sequence data: only one OTU classified as *Holophagae* was observed in treatment LMS_{LMS}.

4.4. Discussion

4.4.1. Examination of LMS_{LMS} and TCS_{TCS} microcosms

With the aim of determining if the bacteria from the TCS site did in fact harbour a more efficient weathering community than was present at the LMS site, measures and comparisons of the bacterial weathering were conducted here. It was apparent that the solutes of microcosms LMS_{LMS} and TCS_{TCS} differed strongly, resulting in two distinct chemical compositions. The same was also true for the bacterial inhabitants of these microcosms as two highly distinct bacterial communities were recorded.

The main differences in taxa abundance between LMS_{LMS} and TCS_{TCS} microcosms were higher abundances of the genera *Veillonella* and *Desulfitobacterium* in the TCS_{TCS} microcosms, whereas, LMS_{LMS} microcosms had a higher abundance of the genus *Geothrix*, a taxon known to be associated with Fe reduction (Coates et al., 1999, Nevin & Lovley, 2002). As with the results of chapter II, the slight, yet statistically significantly, higher pH levels measured from the LMS_{LMS} microcosm were not expected to promote the presence of *Geothrix* spp. The argument used in chapter II to explain the increase in *Acidobacteria* (such as the *Geothrix* spp) from LMS sites was that of oligotrophic habitat preference. This may be true also for the LMS_{LMS} microcosms; however this was not measured here and is purely conjectural. Another explanation may be that the higher pH values, both in LMS_{LMS} microcosms and in the environment, can promote the reduction of Fe in soils (ChengShuai et al., 2010), making it more likely that taxa such as *Geothrix* spp can persist.

The abundance of OTUs affiliated with *Burkholderia* from TCS_{TCS} microcosms, measured from 16S rRNA gene sequences was not obvious from the tRF data. A significant abundance of *Betaproteobacteria* was expected in the TCS_{TCS} microcosms as this taxon is a known root-associated bacterial group, which likely utilises plant-derived organics retained in the soils (Estrada-de los Santos et al., 2011, Keith et al., 2005). Furthermore, this taxon was also previously shown to be important in weathering studies (Lin et al., 2006, Perez et al., 2007).

The most significant difference between the bacterial communities of microcosms LMS_{LMS} and TCS_{TCS} was due to the high abundance of *Firmicutes* detected in the TCS_{TCS} microcosms. The phyla *Firmicutes* include phylogenetic classes such as *Clostridia*, *Bacilli* and *Mollicutes* (Wolf et al., 2004). *Mollicutes* do not possess a cell wall and are parasitic (Wolf et al., 2004), *Bacilli* are aerobic or facultative anaerobes (Wolf et al., 2004), whereas *Clostridia* in general are anaerobic and have been observed as important anaerobic Fe reducers in industry (Venkata Mohan et al., 2011). The examination of bacterial communities from microcosms in this chapter showed that the phylum *Firmicutes* contributed substantially to the differences observed between LMS_{LMS} and TCS_{TCS} soils, with TCS_{TCS} microcosms showing a significantly higher abundance than LMS_{LMS} microcosms. The phylum *Firmicutes* has recently been amended to include a new class, *Negativicutes* (Marchandin et al., 2010), which have been associated with decay and apatite weathering in human dentition (Ribeiro et al., 2011), although a specific role in the weathering process was not established. However, as the chemical composition of dental structures is predominately hydroxylapatite, this inferred relationship between *Negativicutes* and the weathering of human dentition may be important in

the weathering of other apatite mineral from soils in the natural environment. This new class incorporates the family *Veillonellaceae* and genus *Sporotalea*. The family *Veillonellaceae* has specifically been identified as capable of significant Fe reduction in soils (Li et al., 2011), and their abundance in treatment TCS_{TCS} is likely to have been a result of the high availability of Fe in the soil and the water column (section 4.3.1.). One dominant member of the *Firmicutes* in treatment TCS_{TCS} was the family *Peptococcaceae*, which has the potential play a significant role in the cycling of Fe within the microcosms; a strategy observed previously in Fe-reducing media (Abu Laban et al., 2010).

4.4.2. Liberation of solutes from substrate

Comparison of the mean raw data indicated that total Fe in the aqueous phase of the microcosms was 80 times higher in treatment TCS_{TCS} than LMS_{LMS}; although, this was not statistically significant due to high variance in the data. This level of Fe may have sustained the significant *Firmicutes* population observed in treatment TCS_{TCS}, as Fe concentration has a well-defined relationship with the abundance of this taxon (Gihring et al., 2011).

Images of the flasks used for the microcosm experiments suggest that there is substantial Fe oxidation occurring (Figure 4.6) within the microcosms. Several Fe³⁺ deposits were observed but not examined in the course of this research. Therefore, without any reinforcing statistical data, claims regarding the processing of Fe are merely suggestive of trends.

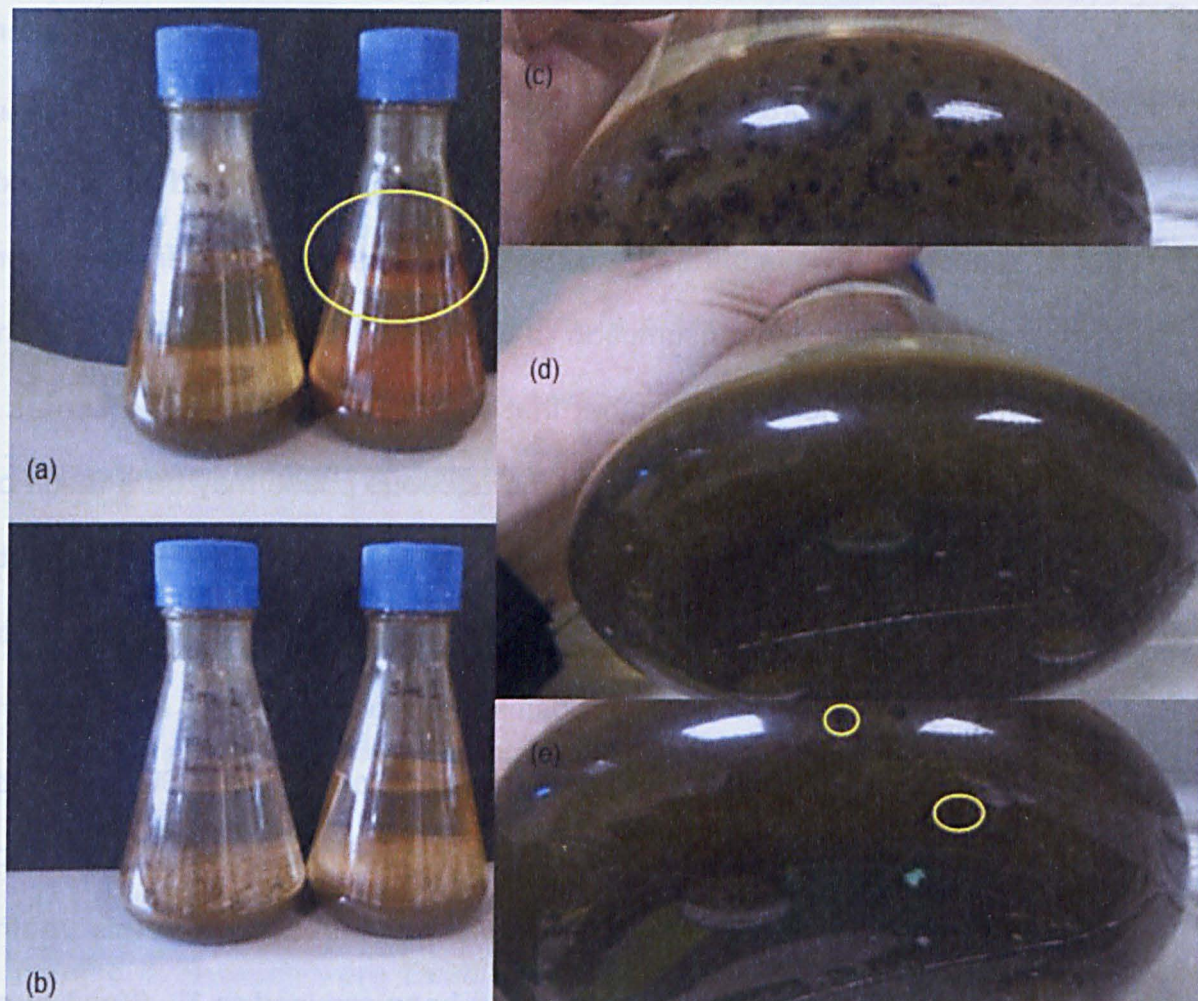


Figure 4.6: Fe banding and spotting of experimental flasks. (a) Shows typical treatment TCS_{TCS} flasks, with abiotic control on left side and biotic sample on right. Note the highlighted Fe band at water surface layer. (b) Shows typical flasks from treatment LMS_{LMS} with no Fe banding present; with abiotic control on left side and biotic sample on right. (c) Presents the most abundant occurrence of Fe spotting from all treatment MIX_{TCS} flasks. (d) Indicates treatment MIX_{CT} , an abiotic control and shows no Fe spotting. (e) Indicating the most numerous spotting apparent from all treatment MIX_{LMS} flasks; here three individual spots were visible. Two have been highlighted.

Five of the biotic solute liberates differed between treatments LMS_{LMS} and TCS_{TCS} (Table 4.4); these were pH, Cu, Si, NO_3 and SO_4 . Similar to the TCS environmental samples in chapter II (section 2.3.1), treatment TCS_{TCS} microcosms

exhibited a lower pH value. This was unexpected because the buffering capacity, measured by gran alkalinity, was higher than that in treatment LMS_{LMS} (Table 4.2), although not statistically significant (Table 4.4). Cu and Si also exhibited lower concentrations in treatment TCS_{TCS} microcosms; the lower Cu concentrations may be explained by consumption of the metal ions by bacteria, with the metal ions binding to the cell wall rather than entering into solution (de Lurdes et al., 1987). The lower Si concentrations in the aqueous phase may be a result of the high Fe concentrations in the solution (Yee et al., 2003), as Fe is capable of abiotic mineralization of Si resulting in an insoluble mineral that can precipitate out of the aqueous phase of the microcosms, therefore would not have been measured by these methods. The lower concentrations of NO₃ and SO₄ in treatment TCS_{TCS} microcosms are potentially explained through nitrate and sulphate reduction by the high abundances of *Desulfitobacterium* and *Veillonella* in this treatment and it is possible that these compounds had been reduced to N₂ (Kapil et al., 2010) and sulphide (S²⁻), respectively. Conversely, the higher concentrations of NO₃ and SO₄ in the LMS_{LMS} microcosms may be explained by the presence of heterotrophic bacteria which use these compounds as electron receptors (Hunter et al., 1998). Furthermore, assimilation of these compounds by microorganisms for use in protein and amino acid production has been reported (Nicholas, 1967); however, none of these proposed mechanisms, and resulting products, could be detected by the methods described in this chapter.

4.4.3. Error in experimental design

Due to the chemical and/or organic differences in the soil matrix (Chapter II, XRF data) used as the substrate in the microcosms, no direct comparison of data from LMS_{LMS} and TCS_{TCS} microcosms can be made other than speculative claims and potential hypotheses to explain the results obtained. An attempt to redress this was made by performing the experiment a second time, using a composite soil (MIX soils). From this experiment it was intended that a direct comparison of the any potential differences between liberated solutes and bacterial composition could be used to infer microbial weathering in soils.

4.4.4. Composite soils

The differences in bacterial community composition between treatments MIX_{TCS} and MIX_{LMS}, measured by tRF analysis, were predominately due to an abundance of *Clostridia* and *Alphaproteobacteria* in the MIX_{TCS} microcosms, the most numerous of which were *Desulfitibacter spp* and *Caulobacter spp*, respectively. This group of *Alphaproteobacteria* are key agents in several studies of weathering (Berestovskaya et al., 2006, Stretch & Viles, 2002) and were observed in high abundances at the TCS sites during the pilot stages of this thesis. However the confidence in the results of the pilot data were low due to poor consistency of 16S rRNA gene sequences obtained from external sequencing supplier. Therefore, the data was not included in this thesis other than the notation here.

Although there were observed differences in bacterial community between the MIX_{LMS} and MIX_{TCS} microcosms, there were no statistical differences overall between microcosms for either chemical solutes liberated or the bacterial communities identified therein. It is conceivable, that the organic content and

mineral composition of each soil has shaped the bacterial community structure, as was inferred from data in chapter II. Indeed, Tiedje et al. (2001) suggested that the diversity of organic compounds in soil was a driver of microbial diversity. As much of the organic carbon from the samples would have survived the sterilization methods employed (Shi et al., 2010), the bacterial communities may have been responding to the organic characteristics of the soils, rather than the soils altering under the influence of the microbial communities. The differences in bacterial α -diversity observed between microcosms in this study (Figure 4.4) potentially corroborate this finding. The mixture of organics from both LMS and TCS sites would be expected to generate a higher organic diversity as the organics from either site would have been generated via different processes. For example, the TCS site soils would be rich in organics derived from leaf litter decomposition, higher cellular biomass (Chapter II) and root exudates, whereas the soils from the LMS site would have organics derived from aerial deposition of microorganisms, higher diversity of microorganisms (Chapter II) and lichen associated organics at the soil surface. Therefore, the organics from composite soils, MIX_{TCS} and MIX_{LMS}, are likely to be more diverse than their individual constituents and may increase bacterial α -diversity within the microcosms (Tiedje et al., 2001). However, as the organics were not characterised here this is merely a speculative potential explanation.

This chapter used modern methods of microbial ecology to develop a greater understanding of bacterial communities and their weathering capability *in-vitro*. It combined molecular microbial analysis and chemical solute liberation measurements in order to examine the role of bacteria in biotic weathering of soils

from the sub-surface critical zone. The role of bacteria in weathering has previously been investigated using isolated bacteria *in-vitro* (Chapter III of this thesis; Barker et al., 1998, Calvaruso et al., 2006, Puente et al., 2004, Uroz et al., 2007). In addition, general soil bacterial communities have been extensively studied *in-situ* (see Janssen, 2006 for a review). However, bacterial weathering by whole communities *in-situ* has seen a paucity of knowledge (Hiebert & Bennett, 1992, Palmer Jr & Hirsch, 1991). This study has shown that *in-vitro* examination of whole community weathering efficiency is still a difficult task and the results remain conjectural at present.

4.5 Limitations to this study

This chapter addressed three simple hypotheses; however the complex nature of the experiments and the communities therein restricted any clear interpretations that could have been made. For example, H8 was designed to determine the effect that the bacterial community would have on the soils they inhabit. However, the chemical composition of the microcosms appeared to alter the bacterial community instead. This resulted in highly dissimilar communities for the LMS_{LMS} and TCS_{TCS} trials and highly similar communities for the MIX_{LMS} and MIX_{TCS} trials, none of which yielded data sufficient to answer the stated hypothesis. The LMS_{LMS} and TCS_{TCS} trials differed in the initial soil composition and therefore were not comparable. The MIX_{LMS} and MIX_{TCS} trials did share comparable soils substrates, however the alteration of the bacterial community resulted in highly similar solute liberates being measured. Although these data are valid, a poor *a priori*

understanding of the microcosms resulted in an experimental design that was unable to address the stated hypothesis with any confidence.

The aim of hypothesis H9 was to determine the effect that the presence of bacteria had on the solute liberation to the aqueous phase. This was not an original aim of the experimental design. To achieve this, an equal number of biotic treatments and abiotic controls would have been required. However the abiotic controls in this chapter were designed to ensure that no contamination was present in the treatment microcosms. The result of this was an unbalanced comparison between biotic and abiotic solutes. Statistical analysis of a design of this nature was possible; however the high variability in the data would dictate that a larger number of replicates with corresponding controls would be needed to have significant confidence in the results. These data presented here offer an indication that biotic weathering did occur yet confidence in the results low.

The final hypothesis examined here (H10) was to determine if individual chemical species could be correlated to bacterial abundance. The convergence of bacterial communities for MIX_{LMS} and MIX_{TCS} restricted this approach. To obtain a clear result, any differences in bacterial community and individual chemical solute liberated were compared. As the MIX soils were similar pre- and post-incubation and the bacterial communities converged, there were little differences between data sets from either site for comparison. The significant correlation presented here for the LMS_{LMS} and TCS_{TCS} trials were viable, however the confidence was low due to same unbalanced approach, which impinged hypothesis H8.

4.6. Conclusions

The results of this study indicate that the bacterial diversity of the samples following incubation may be influenced by the chemical and/or organics of the soil matrix in which the community was incubated, rather than the bacterial composition of the inocula altering the soil substrate (H8). The abundances of a number of bacterial groups in treatments LMS_{LMS} and TCS_{TCS} were likely driven by metabolic processes linked to chemical compounds measured from the water column. Increased concentrations of SO₄ and NO₃ were mirrored by higher abundances of *Desulfitobacterium* and *Veillonellaceae*, respectively (H10). Moreover, bacteria closely associated with Fe (*Veillonellaceae* and *Geothrix*) were present in high abundances in treatment TCS_{TCS}, which had high concentrations of Fe_t (H10).

The treatments MIX_{TCS} and MIX_{LMS} used homogeneous composite substrates and as a result, the bacterial communities were highly similar, with similar chemical or biological characteristics. However, the mixture of LMS and TCS soils would have resulted in a more diverse chemical and organic signature in these treatments and as a result bacterial diversity was higher in these treatments compared to the individual soils.

An initial aim of this chapter was to determine the effects of the bacterial community on weathering. Due to low power in the statistical analysis, this aim cannot be addressed. The data presented in this chapter is suggestive that the presence of bacteria at the sub-surface critical zone may enhance weathering but any claims would be speculative (H9).

It is clear from these results that the sub-surface critical zone may harbour a diverse bacterial community that is driven by the environment more than the environment is altered by the bacterial community. These data presented in this chapter, support the claims in chapter II that the organics derived from higher plants at TCS sites and that metal liberated during weathering (Chapter III) may be influencing the bacterial diversity at the sub-surface critical zone.

Chapter V

Bacterial necromass as a source of organic carbon at the critical zone

5.1. Introduction

Soil organic carbon (SOC) is the largest terrestrial pool of carbon in the world with a substantial contribution to the world's carbon cycle with around 1500 Pg of carbon is stored in this terrestrial sink (Malhi, 2002). Critically, the stability and sustainability of these carbon pools is closely linked to the microbial communities that inhabit soils, as microbes have a strong influence on the SOC dynamics (Waldrop & Firestone, 2004).

Critical relationships between soil microbes and SOC have been reported across a wide range of soils, from arid deserts to arable soils (Allison, 1973, Allison et al., 2010, De Graaff et al., 2010). For example, SOC varies with depth and soil type such that soil just a few mm beneath the surface can have a unique chemical composition, when compared to that of the soil crust (Chorover et al., 2007). Furthermore, the presence of plants and root systems generally increases SOC within the soil matrix. However, rhizospheric influence diminishes once the physical presence of root structures is no longer obvious, as deeper samples tend to be lower in SOC than surface layers (Jobbágy & Jackson, 2000).

Changes in the SOC concentrations of soils can vary with other environmental factors. For example, SOC has a clear relationship with temperature (Jobbágy & Jackson, 2000), such that cold Icelandic soils are expected to be limited in SOC compared to more temperate environments. This is evident in Western Iceland where cold unstable soils were measured as having low SOC pools (Arnalds, 2004, Oskarsson et al., 2004).

Given that carbon can be a limiting factor in microbial communities (Lynch & Whipps, 1990), microbes in these carbon-poor environments may have a

significant dependence on carbon which has been sequestered by other organisms. Microbial biomass from all domains (Archaea, Bacteria and Eukarya) may therefore be a substantial carbon pool in soil ecosystems. Moreover, the presence of root-forming plants can supplement the SOC pool as well as affect the microbial biomass, as plants and microbes can have a significant relationship. As observed in chapter II, higher plant presence influenced bacterial diversity (α and β) and increased cell biomass. Furthermore, the microbial community can shape the sub-surface critical zone chemistry through the liberation of essential elements and nutrients (Chapters III and IV) along with the sequestration of organic and inorganic carbon (Grayston et al., 1998).

As previously discussed in this thesis, the nutrients available at the sub-surface critical zone may influence microbial diversity (Chapter IV). Hunter et al. (1998) discussed the potential for carbon to be a limiting factor to microbial growth at the soil sub-surface as well as the importance of the minerals and elements liberated from weathering as drivers of microbial redox reactions in the soil sub-surface. They argued that lower pO_2 observed in deeper soils resulted in the heterotrophic oxidation of alternative electron acceptors such as NO_3 , SO_4 , Fe (III) and Mn (IV). The results obtained from chapter IV partially corroborate this claim as the predicted anaerobic environment from microcosms was thought to be responsible for increased concentrations of SO_4 and NO_3 which in turn were mirrored by higher abundances of *Desulfitobacterium* and *Veillonellaceae*, respectively.

The likelihood of a heterotrophic bacterial community dominating the sub-surface critical zone has been reported previously (Akob & Küsel, 2011, Chorover et al., 2007). Although Akob and Küsel (2011) noted that there is a rich taxonomic

diversity at the sub-surface, functional diversity was limited to two trophic strategies, chemolithotrophy and heterotrophy. However, identification of the trophic behaviours stated by Akob and Küsel (2011, and references therein) was mostly achieved through comparisons of taxonomic identity to known trophic groups, as the potential carbon sources available to bacteria were not determined empirically.

The sub-surface critical zone at the site inhabited only by lichen and mosses (LMS) was exposed to lower temperatures compared to the sub-surface critical zone at the site inhabited by higher plants (TCS; see Chapter III), contained no visible root system, was lower in SOC than TCS site (Chapter II) and the compounds and elements described by Hunter et al. (1998), thought to be electron acceptors that may drive heterotrophic diversity, were present. Therefore, it was important to discover what was driving the higher bacterial diversity recorded for LMS sites in Chapter II and whether heterotrophic oxidation, as proposed by Hunter et al. (1998), was potentially responsible. Furthermore, it is important to discover if heterotrophy at the sub-surface critical zone is the dominant bacterial survival strategy or whether alternate carbon strategies, such as chemolithotrophy, are equally as important.

Recent measures of bacterial heterotrophy involved tracers such as amino acids (Coelho-Souza et al., 2012, Kirchman et al., 2009). However, rather than restrict this study to a small portion of the carbon available at the sub-surface critical zone, the use of bacterial necromass was employed. Cell necromass has been used successfully in previous studies for enriching the microcosms of biologically active soils (Kindler et al., 2009), as it represented a true heterotrophic source of carbon

within a turnover assay using stable isotopes. Here, a similar approach was adopted to assess food web dynamics using DNA-stable isotope probing (DNA-SIP; Lueders et al., 2003, Neufeld et al., 2007, Radajewski et al., 2000) to detect the individual taxa capable of utilizing cell necromass as a carbon source.

5.1.1. Aims

The immediate aims of this research were to determine if bacterial necromass was a potential source of carbon at the sub-surface critical zone and whether it was capable of sustaining, to some degree, the heterotrophic bacterial community thought to be present at the sub-surface critical zone in the LMS site.

To achieve this, isotopically labelled bacterial necromass was selected as a traceable label for enriching microcosms of biologically active soils, as it represented a realistic heterotrophic source of carbon. This approach permitted the flow of carbon through a bacterial food web to be monitored (Radajewski et al., 2000) to address the following hypotheses:

H11. A heterotrophic bacterial community inhabits the sub-surface critical zone

H12. Bacterial necromass is a potential source of carbon used by bacteria at the critical zone.

5.2. Materials and methods

5.2.1. Sample sites

Microcosms were established containing bacterially active soils from site LMS1 sub-surface critical zone. Soil samples were collected in June 2010 (c. 500 g samples, $n = 9$). A full description of sampling regime and sample site is given in Chapter II. All soils were pooled together for the purpose of this experiment.

5.2.2. ^{13}C labelling of necromass

The initial stages of the research involved incorporating an isotopic label into the cellular biomass of bacterial isolates. Six isolates were selected at random (AW1 4, AW3 13, S1 21, S1 30, S3 6 AND S3 29b; see Chapter III, Table 3.4), pooled together and cultured in 50 mL of M9 mineral media (Sambrook et al., 1989), containing 0.4% (w/v) fully labelled 99 atom% ^{13}C -labelled glucose (Goss Scientific, Cheshire, UK). Glucose was the sole carbon source used. Cultures were incubated for six days at 15°C at 100 rpm. Cells were harvested by centrifugation at 14,100 $\times g$ for 30 minutes then rinsed with 1X phosphate buffer solution to remove any residual media containing ^{13}C -labelled glucose. All isotopically labelled isolates were pooled and suspended in 200 mL of sdH_2O and autoclaved for 15 minutes at 121°C; the resulting necromass was stored at -20°C.

5.2.3. ^{13}C delivery to bacterial community and harvesting regime

To deliver the isotopically labelled necromass to the bacterial communities, microcosms ($n = 2$) were prepared in Erlenmeyer flasks using 50 g soil (wet weight) containing viable environmental bacterial communities and 5 mL of ^{13}C

labelled bacterial necromass suspension. Microcosms were incubated in a stationary state at 5°C for 21 days. This temperature was chosen as it approximated the mean annual temperature experience by the bacterial community *in-situ* (Chapter III). Aliquots of soil (2 g) were harvested from each microcosm immediately following inoculation (t_0) then again after 24 hours (t_1), 72 hours (t_2) and 504 hours (t_3). All aliquots taken were sealed and stored at -80°C prior to functional microbial analyses using DNA-SIP.

5.2.4. Nucleic acids extraction

All total nucleic acid extractions were carried out using the method described by Griffiths et al. (2000), with 1 μ L GlycoBlue (Invitrogen, Paisley, UK) added during the PEG precipitation phase to enhance visibility of the nucleic acid pellet following centrifugation.

5.2.5. DNA-Stable isotope probing (DNA-SIP)

To determine incorporation of isotopically labelled carbon into the DNA of bacteria utilizing cell necromass, nucleic acid extractions were subjected to isopycnic ultracentrifugation as described by Neufeld et al. (2007). In brief, 270 – 460 ng of nucleic acid were added to a 5.5 mL CsCl gradient solution and adjusted to a density of 1.725 g mL⁻¹ using a gradient buffer. Samples were centrifuged at 177087 xg for 50 hours at 20°C using an Optima™ L-90K ultracentrifuge (VTi 65.2 rotor, Beckman Coulter, High Wycombe, UK) with maximum acceleration and no brake settings applied. Following ultracentrifugation, gradient tubes were fractionated from below by water displacement into 12 fractions of 400 μ L CsCl + DNA solution. DNA was retrieved by PEG precipitation with 1 μ L of GlycoBlue

(Invitrogen, Paisley, UK) added. Precipitated DNA pellets were washed in ice cold 70% EtOH to remove residual salts and then resuspended in 30 μ L of sdH₂O. All nucleic acids obtained from density gradient fractions were stored at -20°C.

5.2.6. Determination of fractions containing isotopically labelled DNA

DNA-SIP experiments generally target a specific organism and/or labelled substrate (Lueders et al., 2003, McDonald et al., 2005), resulting in a clean banding of labelled (heavy) and unlabelled (light) DNA. However, this study used a mixed bacterial community and a non-specific carbon source; hence it was possible that a large proportion of bacteria incorporated the isotopic label resulting in overlapping heavy and light DNA between fractions, due to the variability in buoyant density of the bacterial community's DNA. To determine which fractions contained only heavy DNA, it was necessary to both measure and calculate the density distribution of all potential DNA fragments from the bacterial community. Measured densities of each fraction from the blank control were used to plot and confirm that successful density gradient formation was achieved. In addition, calculated DNA distribution along the established density gradient was predicted using GC content of partial 16S rRNA sequences from the bacterial community (NG sequences obtained in chapter 2). These predicted distributions were visually compared to the observed distribution of DNA obtained from ¹²C control tubes, to determine the relative distribution of DNA containing only ¹²C.

To achieve this, it was first necessary to determine the buoyant density of the DNA from the general bacterial community. The mole fraction of guanine + cytosine (GC) content of the bacterial community was calculated from 16S rRNA gene

sequences as per Rice et al. (2000; sequences used were obtained in Chapter II) and the buoyant density of each sequence was calculated using the formula reported by Schildkraut et al. (1962):

$$p = \frac{(GC * 0.098)}{100} + 1.66$$

Where GC represents the mole fraction GC content of each separate sequence and p represents the density of each sequence in g mL^{-1} . As labelled DNA is less buoyant than unlabelled DNA, the density of a given sequence will increase by 0.036 g mL^{-1} for fully labelled sequences (Birnie & Rickwood, 1978). Using the calculated densities for unlabelled sequences and predicted densities of fully labelled sequences, the DNA distribution between fractions was derived. Each fraction's density was measured from (1.725 g mL^{-1}) control centrifuge tubes, containing no DNA, that were subjected to isopycnic centrifugation with treatment and ^{12}C control tubes. Control tubes containing DNA without ^{13}C enrichment were fractionated as per treatment tubes and the presence of DNA in each fraction was confirmed using agarose gel electrophoresis (1%), which were visualized by ethidium bromide staining.

5.2.7. Terminal restriction fragment length polymorphism (tRFLP) and *in-silico* digestion and identification

Selected heavy and light DNA fractions were amplified and tRFLP analysis carried out using the methods described in Chapter II. Approximate classification was assigned to each tRF using the criteria outlined in Chapter II. The 16S rRNA gene

sequences used for in-silico digestion and subsequent taxonomic identification of the tRFs were generated in Chapter II (section: 2.2.5.).

5.2.8. Data analysis

tRFLP data were analysed for differences in tRF presence between heavy and light fractions by analysis of similarity (ANOSIM; Clarke & Warwick, 2001). Principle components analysis (PCA) was used to visualise the tRF distributions of heavy and light fractions and to compare the bacterial communities therein with the LMS site bacterial community described from chapter II.

5.3. Results

5.3.1. Fraction determination

Control tubes containing ^{12}C DNA without ^{13}C enrichment showed evidence of DNA present in fractions 6 – 12 (Figure 5.1). Therefore, fractions one, two and three were considered to be heavy fractions as there was no ^{12}C labelled DNA, either calculated or measured, found to be present within. Fractions seven, eight and nine were considered to be light fractions as these consistently harboured ^{12}C labelled DNA.

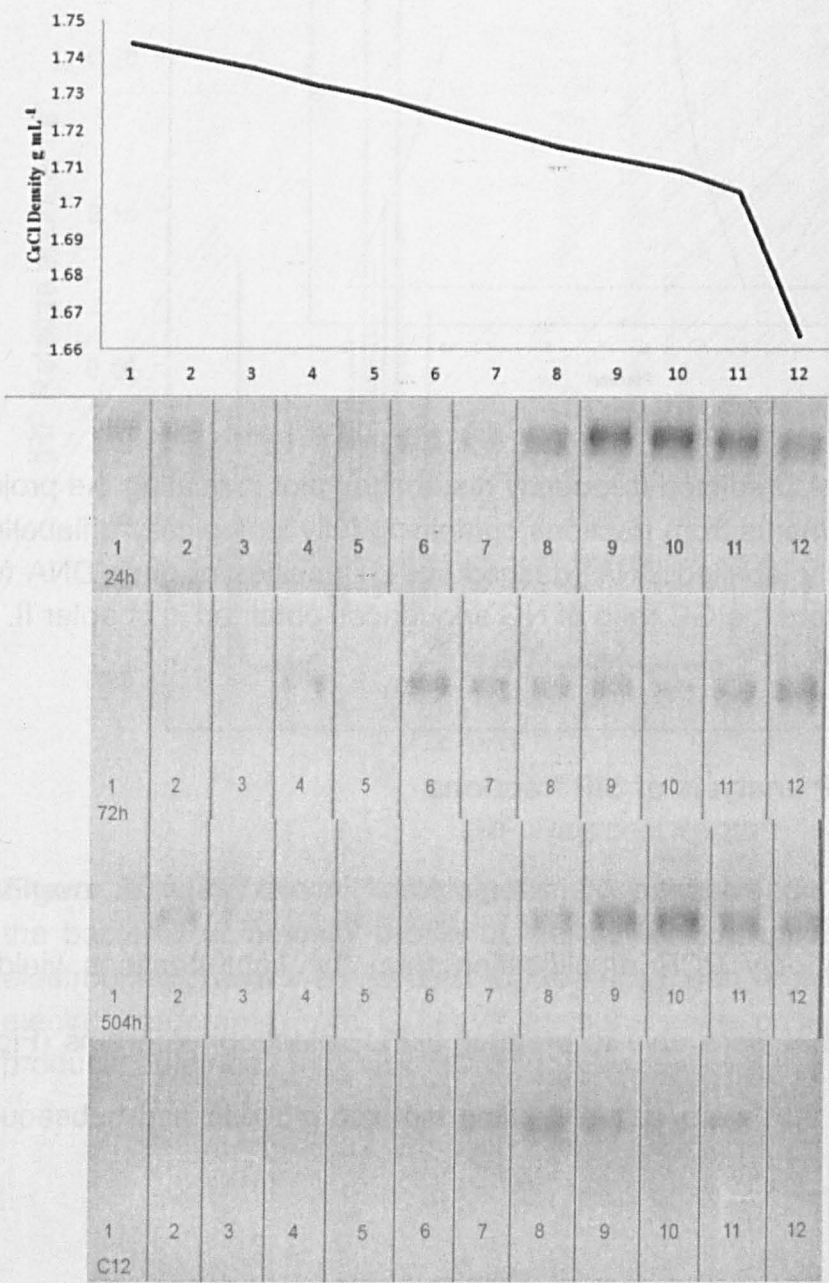


Figure 5.1: The gradient formation plot, as measured from the blank control tube, is presented. Below this are agarose gels indicating the PCR amplicon banding of sample tubes from ^{12}C control, 24, 72 and 504 hour incubations. There were no PCR amplicons evident in the ^{12}C control below fraction number 6.

Note the reduction in density for the 12th fraction. This is caused by the addition of sdH₂O used to displace the CsCl gradient during fractionation.

Due to the diverse bacterial community expected from these incubations the separation of ^{12}C and ^{13}C enriched nucleic acids was not distinct across the gradient tubes. This was explained through the GC content of the DNA of the community increasing the variability of DNA buoyancy (Figure 5.2).

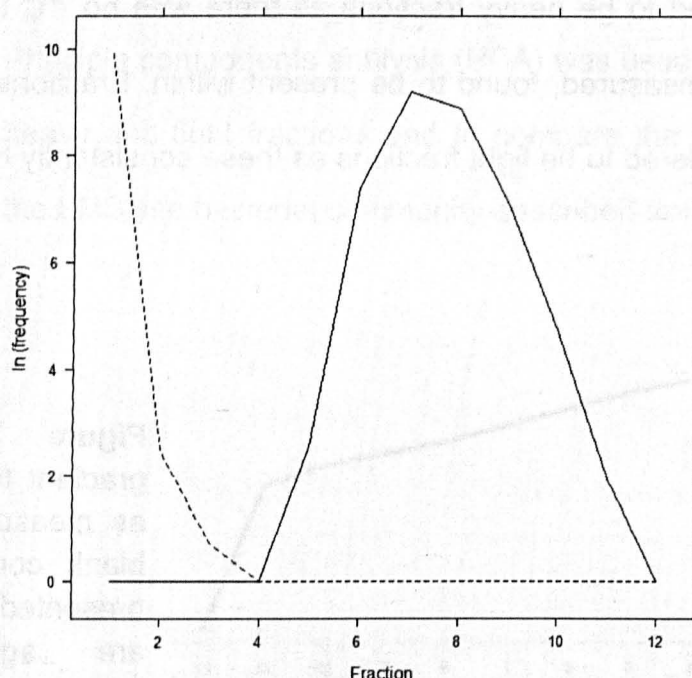


Figure 5.2: A predicted frequency distribution plot indicating the projected number of DNA fragments from fractions containing fully isotopically unlabelled DNA (solid line) and fully labelled DNA (dashed line). Densities of each DNA fragment were calculated from the GC ratio of NG sequences obtained in chapter II.

5.3.2. tRFLP analysis of SIP fractions

There was no evidence of isotopic label incorporation or overloading from t_0 samples as only PCR amplification from the light fractions yielded any PCR amplicons that were able to produce tRFLP electropherograms (Figure 5.3). The six isolates that were cultured using isotopic glucose and subsequently used as

isotope inocula were not detected in the t_0 sample. Of the six isolates used as the ^{13}C inoculum, only one (S3 29b) would have yielded a fragment < 300 n.t. in length using the *MSP1* restriction endonuclease site; this *Pseudomonas* spp isolate would have yielded a fragment of c.126 n.t. in length. Although this was not observed empirically, it was calculated using 16S rRNA sequences obtained in chapter III.

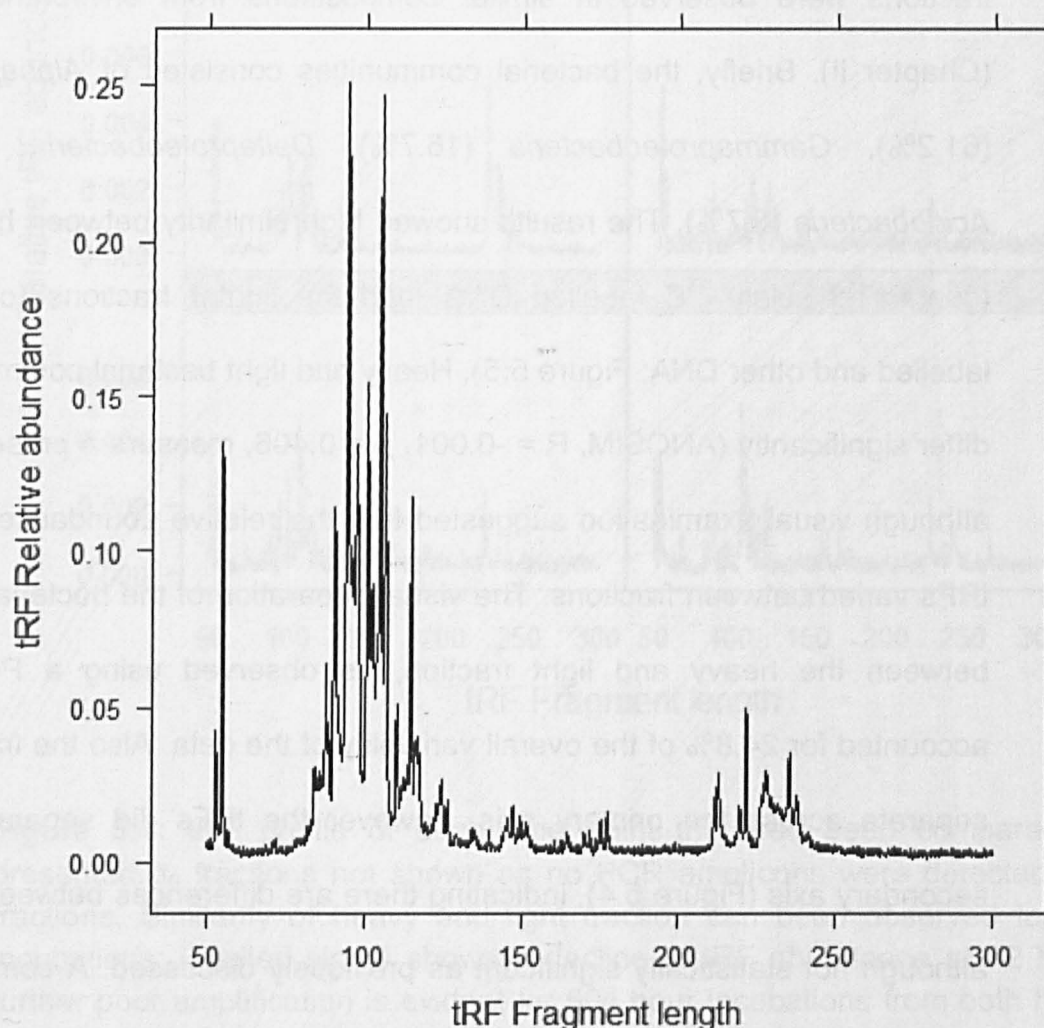


Figure 5.3: tRFLP electropherogram of t_0 sample (light fractions). This indicated the bacterial community profile at the initiation of incubations. All light fractions electropherograms were pooled to create a mean representation, used here. No electropherograms from t_0 heavy fractions were possible due to lack of PCR products obtained.

tRFLP analysis to determine the genetic fingerprint of the bacterial communities utilising the ^{13}C -labelled necromass showed that the label was incorporated into bacterial DNA within 24 hours of incubation (Figure 5.4). Overall, c. 90% of tRFs determined in samples collected after 24 hours had incorporated the isotopic label. Taxonomic identification of tRFs revealed that >93% of tRFs identified in the fractions were observed in similar compositions from environmental samples (Chapter II). Briefly, the bacterial communities consisted of *Alphaproteobacteria* (61.2%), *Gammaproteobacteria* (16.7%), *Deltaproteobacteria* (10.4%) and *Acidobacteria* (5.7%). The results showed high similarity between heavy fractions (containing solely ^{13}C labelled DNA) and the lighter fractions (containing ^{13}C -labelled and other DNA; Figure 5.5). Heavy and light bacterial communities did not differ significantly (ANOSIM, $R = -0.001$, $p = 0.406$, measure = presence/absence) although visual examination suggested that the relative abundances of individual tRFs varied between fractions. The visual separation of the bacterial communities between the heavy and light fraction, as observed using a PCA ordination accounted for 24.8% of the overall variability of the data. Also the fractions did not separate across the primary axis, however the tRFs did separate across the secondary axis (Figure 5.4), indicating there are differences between the fractions although not statistically significant as previously discussed. A comparison of the heavy and light fractions' bacterial communities against the environmental LMS bacterial community (Chapter II) showed that neither fractions community were distinguishable from a true environmental bacterial community (Figure 5.6).

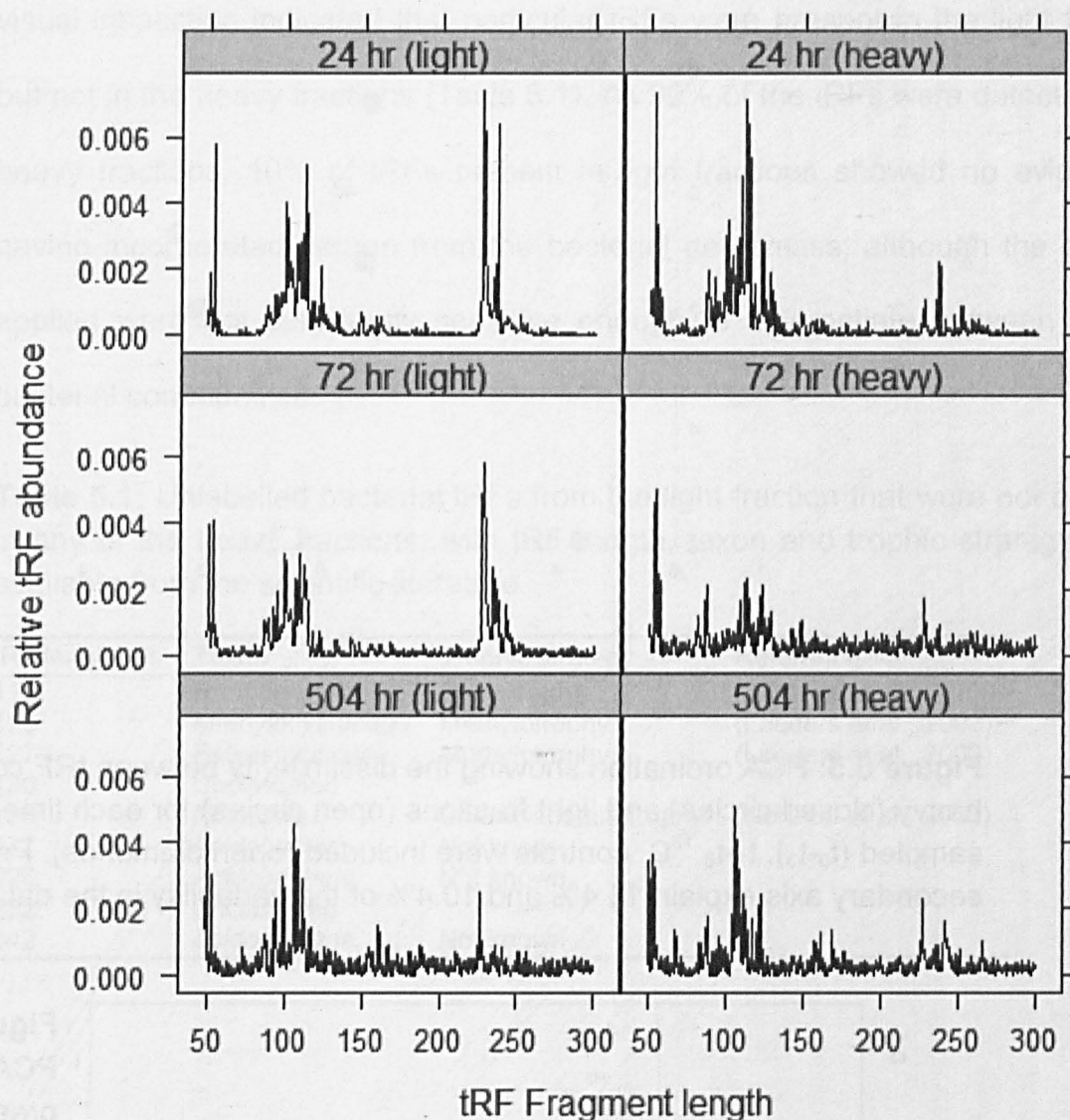


Figure 5.4: tRF profile of each time point that had been compared; means presented. t_0 fractions not shown as no PCR amplicons were detected in heavy fractions. Similarity of heavy and light fraction can be observed for 24 hour incubations; labelled signal shows a decline in tRF abundance at 72 hours and further poor amplification is evident for 504 hour incubations from both heavy and light fractions.

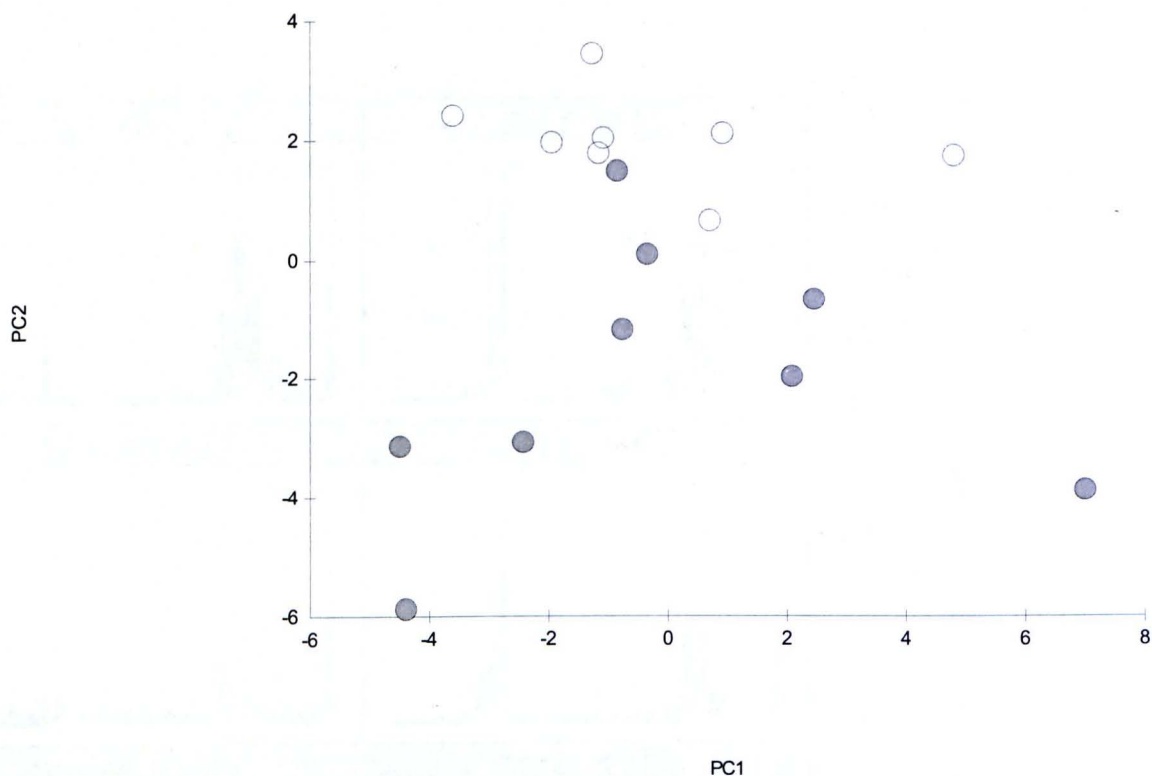


Figure 5.5: PCA ordination showing the dissimilarity between tRF composition of heavy (closed circles) and light fractions (open circles) for each time period sampled (t_0 - t_3). t_1 - t_3 ^{12}C controls were included (open diamonds). Primary axis and secondary axis explain 14.4% and 10.4% of the variability in the data, respectively.

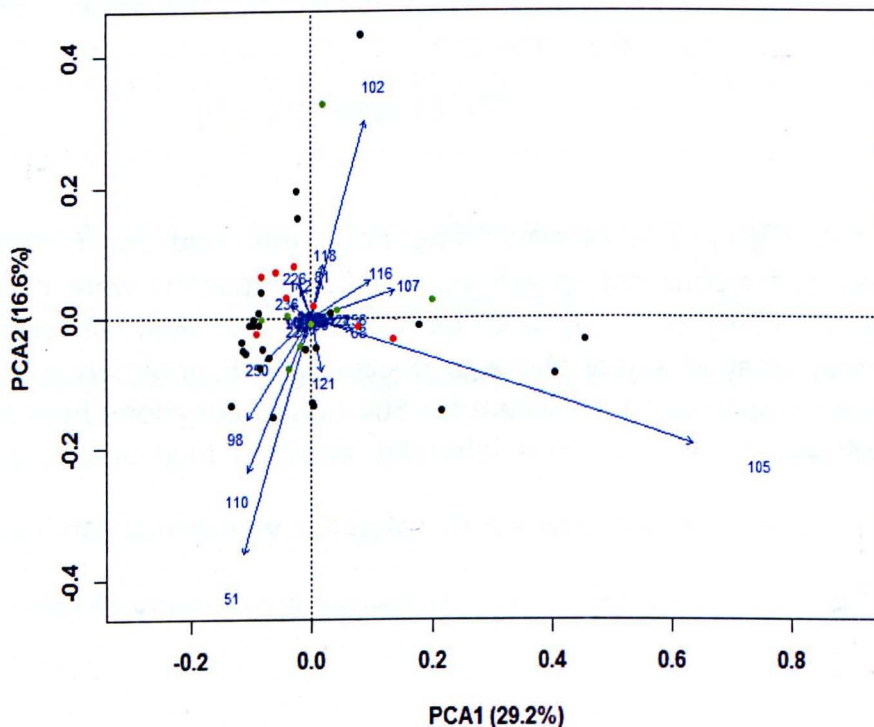


Figure 5.6: A PCA ordination presenting the high similarity between labelled (•), unlabelled (•) and environmental bacterial communities (•). Environmental data was obtained from samples collected in the LMS sites, described in chapter II.

Although the tRF results from heavy and light fractions did not differ significantly, a visual inspection indicated that particular tRFs were present in the light fractions but not in the heavy fractions (Table 5.1). As 90% of the tRFs were detected in the heavy fractions, 10% of tRFs present in light fractions showed no evidence of having incorporated carbon from the bacterial necromass; although the statistics applied were not sufficiently sensitive enough to differentiate between the two bacterial communities.

Table 5.1: Unlabelled bacterial tRFs from the light fraction that were not observed in any of the heavy fractions; with tRF length, taxon and trophic strategy, where available from the scientific literature.

tRF length n.t.	Taxon	Trophic strategy	Reference
115	<i>Rhodoplanes</i>	Phototrophy	(Lakshmi et al., 2009)
125	<i>Methylocystaceae</i>	Methylotrophy	(Lueders et al., 2003)
127	<i>Beijerinckiaceae</i>	Methylotrophy	(Lueders et al., 2003)
130	Unclassified		
134	<i>Nitrospira</i>	Chemolithoautotrophy	(Northup et al., 2003)
177	<i>Acidobacteria</i>	Not known	
226	<i>Acidobacteria</i>	Not known	
232	Unclassified		
242	<i>Acidobacteria</i>	Not known	

5.4. Discussion

In this chapter, it was hypothesised that, as SOC was low in concentration at the sub-surface critical zone of the LMS site (Chapter II), the necromass of bacteria might be one energy source driving a heterotrophic bacterial community. To investigate this hypothesis, microcosm experiments were setup using soils from the TCS and LMS sites. The use of TCS soils was made to permit a comparison between sites trophic characteristics, however this was limited by the poor extraction of nucleic acids from these soils (Appendix i and ii) and so this chapter is limited to discussing the LMS site microcosm results alone.

5.4.1 Dividing the bacterial community into fractions

The identification of bacteria capable of utilizing bacterial necromass was determined by the fractionation of isotopically labelled (isotopically heavy) and unlabelled (isotopically light) DNA from the microcosms. In this study the definition of heavy and light fractions must be more specific than is normally observed in the literature for this type of experiment: A heavy fraction contains only heavy DNA from heterotrophic bacteria, capable of incorporating necromass, whereas a light fraction may contain heavy and light DNA from all bacteria present in the microcosms.

The bacterial communities present in both the heavy and light fractions resemble that of the LMS environmental communities measured in chapter II, to the degree that it was not possible to visually differentiate between them (Figure 5.6). This similarity between fractions can be partially explained by the distribution of DNA in isopycnic treatment tubes. Hypothetically, the light DNA will display a normal

distribution across several fractions having a mean density of 1.725 g mL^{-1} and the heavy DNA will normally distribute between fractions having a mean density 1.761 g mL^{-1} . Based on these density differences, there will likely be an overlap of heavy and light DNA within the median fractions. Also, DNA that is partially labelled would have settled within intermediate fractions between the fractions designated heavy and light. For any bacterial community it would be expected that a few individual taxa can be observed in the light fraction as well as the heavy fractions, confirming both their presence in the community and their ability to incorporate the isotopic label, used here as a proxy for heterotrophic function. In this study there appears to be a rich heterotrophic bacterial community as there are no obvious differences between heavy and light fractions, with both exhibiting highly diverse bacterial communities capable of incorporating necromass as a carbon source.

The observed differences in β -diversity of the two fraction types (Figure 5.5) could not be confirmed statistically. While there were sufficient differences between the relative bacteria communities of the heavy and light fractions to provide a visual comparison, these were too infrequent to be statistically tested using the ANOSIM test. As 90% of the tRFs were common to both fraction types it can be proposed that there is a rich heterotrophic bacterial community present in the microcosms tested here. Furthermore, >93% of the tRFs identified from the heavy fractions were also discovered in the LMS environmental samples (Chapter II). This infers that there is also be a rich heterotrophic bacterial community inhabiting the sub-surface critical zone of the LMS site.

5.4.2. The bacteria found in the heavy fractions

Proteobacteria, specifically *Alphaproteobacteria*, are known to be copiotrophic and are generally found in areas rich in SOC, such as those of the TCS sites in chapter II. The observed incorporation of the isotopic label by this group suggests their ability to use necromass as an energy source in addition to plant derived SOC. This ability to employ multiple carbon sources may provide *Proteobacteria* with a competitive advantage over other oligotrophic-suited organisms. The higher volume of SOC measured (Chapter II) and the predicted increase in plant derived organics (Chapter IV) from the TCS site may illustrate how this taxa can maintain its abundance at the TCS sites, given the extreme environmental conditions experienced (Chapter III).

The lower profusion of *Acidobacteria* in the microcosm experiments was noteworthy, as this group dominated the LMS environmental samples (Chapter II) yet was relatively low in abundance here, even though the soils, and bacterial communities therein, were from the LMS site. The oligotrophic habitat preference of this taxon may have resulted in a reduced relative abundance compared to *Proteobacteria* due to competitive exclusion processes in the microcosms. For example, *Acidobacteria* may outcompete *Proteobacteria* in the LMS environment due to the oligotrophic conditions, however in the microcosms the influx of carbon within the necromass may provide a niche for the *Proteobacteria* in which to flourish. The low SOC and cell biomass recorded from the LMS site in chapter II were not replicated in these microcosm experiments, due to the inocula used, and therefore may have affected the *Acidobacteria* abundance.

5.4.3. Bacteria present in the light fractions but not in the heavy fractions

Recent investigations have argued that heterotrophic bacteria dominate at the sub-surface critical zone (see Akob & Küsel, 2011 for a review), but Akob and Küsel (2011) discuss two other major trophic strategies (methylotrophy and chemolithotrophy) that they believe to be present at the sub-surface.

The discovery of potential methylotrophy was inferred by the presence *Methylocystaceae* and *Beijerinckiaceae*. These taxa have been reported in oxic rice field soils and were capable of obtaining carbon from methane rather than SOC (Lueders et al., 2003). In addition to oxic methylotrophy, anaerobic methanogenesis is also common in soils and CH₄ the product rather than the carbon source; any anaerobic niches within the microcosms, due to the addition of a liquid inoculum (Angel et al., 2011), could potentially have aided in the generation of an alternative carbon source to this bacterial group. The only potentially chemolithotrophic bacteria highlighted here was *Nitrospira*. Although this taxa is known to harbour nitrogen cycling capabilities, Northup et al. (2003) also found this group of bacteria in iron rich environments and suggest that they may be an important ecological group with regard to N₂ and Fe cycling. Given the rich Fe deposits known to be within the soils of this study (Chapter II), their importance cannot be underestimated. These two trophic strategies are both represented in this study by tRFs that are unique to the light fractions (Table 5.1; tRFs 125, 127 and 134 n.t.).

The presence of chemolithotrophic bacteria in the light fractions was expected due to the rich nutrient source present within the soils, as shown by the presence of

several various elements and chemical compounds (Chapter II, III and IV) capable of acting either as an energy source and/or electron acceptors for chemotrophic bacteria; for example, in chapter IV there were elevated concentrations of SO_4 measured in the microcosms that were mirrored by an increase in *Desulfitibacter* spp; this can result in the reduction of PO_3 to PO_4 . This is true also of *Geothrix* spp with Fe, *Veillonella* with NO_3 and again *Desulfitibacter* spp with H_2 ; all of which have been discussed in this thesis (with the exception of H_2). These potential chemical cycling bacteria may be important ecological engineers in the environment, yet their significance to weathering cannot be ascertained here.

One particular group present in the light fraction was *Rhodoplanes*. While this is a common soil microorganism, *Rhodoplanes* can include phototrophic members of this taxon (Lakshmi et al., 2009). Its presence in the deep subsurface is surprising since the sub-surface soils and the microcosms lacked any photosynthetically active light source. One explanation for this may be that they have been transported to the critical zone via aerial deposition or hydro-transportation. Another potential explanation of why this taxon was discovered in only the light fractions may be that they are metabolically inactive yet geographically present heterotrophs, however there is no data presented here to determine the feasibility of either of these possibilities.

Although several tRFs affiliated with *Acidobacteria* were identified from solely the light fractions (Table 5.1; tRF:177, 226 and 242 n.t.), this group of bacteria are relatively poorly studied due to their resistance to culture, and little is known about their trophic strategies. In general they are oligotrophic bacteria but the strategic

preferences of those identified here cannot accurately be examined and discussed using these data in this chapter.

5.4.4. Importance of necromass for bacteria in the natural environment

In previous research (Chapter III), it was evident that 91% of cultured bacteria expressed a MPS phenotype. Making the assumption that this physiological behaviour is as wide spread throughout the non-culturable community, it can be tentatively inferred that necromass could be an important driver of bacterial weathering. Given the 90% of bacteria measured here as capable of using necromass as a carbon source, the surmised high occurrence of the MPS phenotype in the environment (Chapter III) may result in a high number of bacterial weathering phenotypes being heterotrophs, using necromass as a source of carbon. However, the lack of metabolic efficacy for these isolates and trophic data for non-culturable bacteria makes this only one potential scenario.

The rapid incorporation (< 24 hours) of the isotopic label from the bacterial necromass to the DNA of the heterotrophic bacterial community within these microcosms indicates that this is an easily assimilated carbon source. However, as bacterial growth is known to be linked to environmental temperature (Ratkowsky et al., 1982), the higher temperatures measured in the environment, during summer months, may further expedite this heterotrophic behaviour. The temperature at which the microcosms were examined here (5°C), was broadly equal to the mean annual temperature of the environmental sites (Chapter III). However, seasonal temperatures at the sub-surface critical zone (Figure 3.3) can be double that of the microcosms. In addition, the optimal growth temperature of the heterotrophic

bacterial isolates (Sub-cultured in Chapter III) was measured as being higher than the 5°C used for these microcosms experiment. This raises the possibility that bacterial necromass may be incorporated into the heterotrophic community at a greater rate *in-situ*, than in this study, during summer months (Chapter III).

Given the bacterial prevalence for warmer temperatures (Chapter III), the generalist weathering function across bacterial taxa (Chapter IV) and the rich heterotrophic bacterial community identified here, it may be possible to speculate that increases in summer temperature can increase growth and metabolism of the bacterial community. In addition, the generalist weathering phenotype may also increase expression with bacterial activity, suggesting that periods of seasonal weathering may be possible at the sub-surface critical zone and these are driven by the organic carbon afforded to the bacterial communities.

5.4.5. Isotope incorporation over a temporal period

The data obtained here indicated that there was a rapid incorporation of the label. However the subsequent temporal data (t_{2-3}), taken over the course of the incubations, was not as clear in identifying the heterotrophic bacterial community. The examination of the bacterial community relative abundances for t_2 and t_3 time points (Figure 5.4) did not appear to have provided a community recognisable as that of the environment communities (Chapter II). For all three time points the heavy and light fractions displayed similarities in tRF presence/absence. However examination across the temporal data indicated that there were differences in tRF abundance, with incubations from 504 hours almost indistinguishable from background.

For DNA-SIP experiments which employ specific carbon sources, there is generally an optimal time point at which to make any analyses. Following this optimal time, bacterial lysis occurs and heterotrophic peaks appear and the noise in any data set increases. As heterotrophic bacteria were to foci of this study this was not an expected caveat to the method. It did however appear that the measured bacterial community was substantially changing over time.

A predicted outcome of measuring necromass incorporation over time was the loss of isotope signal over time. For example, the isotopic label was incorporated rapidly into the DNA of the heterotrophic community; however, it would also have been incorporated into other cellular structures such as proteins and cell walls. Eventually it was expected that the isotopic signal would diminish while the light fraction community signatures would remain stronger. This diminished signal was observed in PLFA-SIP trial conducted on these samples (Appendix v). However the data was not adequate to provide any specifics regarding the bacterial community in this examination and therefore not included in this chapter. It did however show the rapid incorporation of the label into the phospholipids of the cells.

As this experiment was designed to examine the differences in tRF presence between fractions it is not possible to make any conclusions as to why these temporal changes in bacterial community relative abundances occurred. However, some possible explanations would be: 1) the semi-closed environment of the microcosms may have had a build up of toxins that reduced the bacterial biomass, such as the high Cu from the soils (Chapter III); 2) an accumulation of metals over

time may have inhibited DNA extraction and/or PCR giving the appearance of lower bacterial diversity; 3) the bacterial community may have in fact altered to be dominated by an organism that was not detected using the primer set employed in this study (e.g. Fungi or Archaea). All three of these caveats are feasible, however to determine which of these, or other issues, were responsible would require further investigation.

An aspect of this study that cannot be accurately discussed is the potential for slow-growing bacteria to inhabit the microcosms. The 24 hour incubations showed the highest resolution of data and as this resolution reduced over time so too did the potential to identify any slow growing individual taxa, such as *Chloroflexi*, *Acidobacteria* and *Actinobacteria* (Davis et al., 2005). Therefore, any tRFs absent from the heavy fractions yet present in the light fractions may have been from slow-growing organisms that had yet to incorporate the label. This would have resulted in heterotrophic bacteria not being detected in any of the heavy fractions and therefore omitted from any results and further discussion.

5.4.6. Caveats to this method

As with many SIP experiments the incubation period is critical. The DNA-SIP results show that carbon incorporation from bacterial necromass may occur within one day. Hence, this novel use of DNA-SIP can be employed to monitor the microbial growth within the heterotrophic soil ecosystem in a relatively rapid manner. However, since nucleic acids are biomarkers that monitor growth/division of cells, this technique is limited to monitoring those that are growing/dividing and may overlook cells that, although metabolically active, are in a zero growth state

(Novitsky, 1987). For example, heterotrophic bacteria that are non-viable, but present in sufficient abundance so as to be detected, can be falsely labelled as non-heterotrophic, as they will only be measured in the light fractions. Another confounding issue with this method is that of mixotrophy. The term mixotrophy is used to describe organisms capable of both autotrophic and heterotrophic carbon metabolism. The methods here were unable to differentiate between a heterotrophic and a mixotrophic bacteria. Therefore, individual tRFs identified from the light fractions could potentially be mixotrophs employing an autotrophic strategy, under the conditions within these microcosms.

5.4.7. Methods of enhancing this study

The data presented here showed that the incorporation of ^{13}C was possible and that this resulted in a large heterotrophic bacterial community. However the confidence in this data relies heavily on the accuracy of the controls used throughout this chapter. The blank gradient tubes were examined and a gradient was established for all samples. The densities of individual fractions (heavy and light) were not measured and therefore rely on the accuracy of the blank controls and the premise that the treatment gradient tubes established identical gradients to that of the blank tubes. This can be overcome through examining the density of all fractions obtained throughout the study.

A key method of increasing any confidence in this experiment would be to examine the ratio of $^{12}\text{C}:^{13}\text{C}$ for each fraction. This can be achieved through examination of the fractions using gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS). However, in this study this method was not

available for use. This would have confirmed that fractions consisted of heavy and light DNA. Another method of achieving this was to attempt DNA-SIP RAMAN. The RAMAN microscope is capable of detecting the isotopic carbon from each fraction and displaying this as a shift in the spectra. A pilot attempt of this was undertaken in this study, however proved to be unsuccessful. The peaks normally observable from RAMAN spectra of DNA were not identified. The reasons why this failed are not clear; however, the CsCl from fraction may have created salt crystals that inhibited examination DNA from the RAMAN laser (D.Read, Per. Comm.). If this approach was to be attempted again, it would be necessary to purify all DNA prior to RAMAN examination.

As stated, the high occurrence of heterotrophs examined here and the high number of MPS phenotypes observed in chapter III may infer a rich heterotrophic community that may be able to use necromass as a carbon source during weathering. The data presented in the thesis can only infer this relationship; however, future examination of this assumption is possible. It would be possible to re-examine the isolated bacteria grown on the MPS plates generated in chapter III, however in addition to making the MPS plate phosphate limited, the omission of glucose would result in P and C limited MPS plates. Re-running the MPS experiments outlined in chapter III using P and C limited plates that have been inoculated with necromass may provide a more informative data-set on this matter. However, this method relies on the ability to remove all aqueous P from inocula; as this is not practical a robust series of controls would be required to offer any confidence in this approach.

5.5. Conclusions

It was recorded here that the bacterial community inhabiting the sub-surface critical zone were rich in heterotrophic bacteria (H11). The use of DNA-SIP showed evidence of rapid incorporation (within 1 day) of the isotopic label into 90% of the bacterial community measured by tRFs. This indicates that the majority of the bacterial community present at the sub-surface critical zone are capable of utilizing bacterial necromass as a source of organic carbon (H12), fuelling a significant heterotrophic community. This resulted in a heterotrophic bacterial community that was indistinguishable from the environmental bacterial community.

However, the examination of the heterotrophic community over time was less successful. The signal decreased over time and so too did any confidence in the latter data points. This lack of temporal data has limited the confidence of data from this study to those heterotrophic bacteria that are fast-growing and capable of incorporating bacterial necromass within a 24 hour period.

Summary of work done and general conclusions

6.1. General discussion and summary of the work carried out

This thesis had three main aims. These were to **a)** to explore the diversity of the bacterial community inhabiting the sub-surface critical zone from a region in western Iceland, **b)** determine whether any environmental factors may affect the bacterial community and **c)** elucidate what functional role, if any, the bacterial community had on the process of silicate weathering.

6.1.1. Diversity at the critical zone

The data presented in this thesis confirmed that the bacterial communities from an Icelandic sub-surface critical zone were diverse (Chapter II; H1) and that this diversity did in fact differ depending on the land coverage of the surface soils (Chapter II; H3). Examination of the tRFLP and 454 NGS data from both sample sites (Chapter II) indicated that the presence of *Acidobacteria* and *Alphaproteobacteria* were key in determining the general community composition. This was likely due to the oligotrophic and copiotrophic survival strategies employed by the two taxa reflecting the abundance of SOC (Chapter II) at the different sample sites (LaMontagne et al., 2003).

The drivers of soil bacterial communities were discussed by LaMontagne et al. (2003) for sub-surface soils; they concluded that lower SOC abundance at the sub-surface could result in an oligotrophic bacterial community dominating the area. However, the diversity that they measured cannot be compared here as Shannon's diversity indices were used as a measure of diversity from a community profile and, like this study, have inherent methodological errors (Blackwood et al., 2007).

Bacterial communities from TCS sites were expected to harbour a more diverse composition than the LMS sub-surface soils due to the organics afford the site through the root-systems present, however this proved not to be the case as the α -diversity was greater in LMS than TCS sites (Chapter II). The presence of a rhizospheric zone above the TCS critical zone appeared to have resulted in a more specialized rather than diverse community (Chapter II). However a more specialized community does not necessarily result in a more functionally efficient community, as diverse communities harbour a greater functional redundancy (Bell et al., 2005). This lower diversity may inhibit weathering at the TCS critical zone, as it relies on the weathering efficacy of the dominant specialised bacteria taxa present and the metabolic activity therein. This is not the case for the LMS soil bacterial communities as they harbour a more diverse, potentially opportunistic, community. The data obtained in this study does suggest that the organic/chemical composition of the substrate is a key aspect in determining bacterial diversity (Chapter IV), however the organics, although measured in quantity (Chapter II), were not characterised specifically.

The environmental drivers of the observed differences in bacterial diversity could not be ascertained in this study with any confidence (H2). While the chemical composition of the critical zone at the LMS and TCS sites differed, it was not possible to show any correlation between individual bacterial groups and chemical species. The soil matrix is complex in nature and as such the drivers of bacterial diversity are likely to co-correlate making them indistinguishable with respect to bacterial abundance.

6.1.1.1. Following the food

Although the chemical/organic content of the critical zone soils is an important driver of bacterial diversity, the sub-surface critical zone is relatively poor in organics when compared to the soils surface (Akob & Küsel, 2011). This disparity between the soil surface and the sub-surface may be increased, at the TCS site, by the deposition of carbon through leaf litter thatch; however, at the LMS site SOC remained low as there was no obvious floral carbon source. Despite this, the bacterial community showed a higher α -diversity at the LMS site (Chapter II; H3). In a bid to understand what drove the higher bacterial α -diversity at the sub-surface critical zone of the LMS site (Chapter II), the potential organics available to the LMS bacterial community were investigated (Chapter IV). One possible source was from the bacterial community itself; hydro and aerial deposition of bacteria (Huysman & Verstraete, 1993, Jones et al., 2008, Lindemann & Uppel, 1985) in addition to necromass from the resident chemolithotrophic bacteria populations (Akob & Küsel, 2011, Chorover et al., 2007) may be a source of carbon capable of helping to sustain a heterotrophic population at the critical zone. To investigate this further, the flow of carbon from bacterial necromass through the bacterial community was investigated using stable isotope labelling.

It was apparent that the majority of the bacterial community were capable of utilizing bacterial necromass as a source of carbon (Chapter V; H11 and H12). Although, the bacterial communities from the microcosms did not statistically differ from LMS environmental bacterial communities, there was an indication of higher *Alphaproteobacterial* abundance in the microcosms (Chapter V) than was

observed from the environmental LMS site (Chapter II). This was thought to be a direct result of the increase in organic carbon from the necromass inocula. There were, however, a few bacterial groups identified that did not show evidence of carbon obtained through heterotrophic assimilation of necrotic biomass. These bacterial groups were likely to be capable of obtaining alternate sources of carbon in order to promote growth. For example, alternate sources of carbon were available during the microcosm incubations. These were $^{12}\text{CO}_2$ from the headspace gas, and ^{12}C rich soil organics, which were persistent in microcosms and could have been utilized. These ^{12}C rich carbon sources may be metabolised and subsequently generate secondary metabolites such as methane ($^{12}\text{CH}_4$) and carbon monoxide (^{12}CO). In turn, these secondary compounds may be used via other trophic strategies (photo-, chemolitho- and methylotrophic) which would remain undetected in the heavy fractions. In addition, the aerobic nature of the incubations would have limited the detection of obligate anaerobic bacteria in either the light or the heavy fractions, regardless of their trophic survival strategy.

Using the data here, showing the incorporation of bacterial necromass (90% of isolates; Chapter V) and comparing to the high levels of the MPS phenotype from isolated bacteria that were observed in the cultured heterotrophic community (Chapter III), it can be inferred a large contingent of bacteria may enhance phosphate weathering through incorporation of bacterial necromass. However, this makes the assumption that the non-culturable bacterial community share the high occurrence of the MPS phenotype observed from the culturable community, and this is an, as yet, unqualified limitation.

6.1.2. Habitability of the environment

The next challenge for this thesis was to determine if the sub-surface critical zone at the LMS and TCS sites was an environment capable of sustaining optimal bacterial growth. It was observed that most isolates examined showed an optimal temperature for growth that was higher than that experienced at the sub-surface critical zone (Chapter III; H4). There was an indication of psychrotolerant growth, yet no psychrophilic bacterial isolates were identified (Chapter III).

6.1.2.1. Cu tolerance

The effects of Cu on bacterial growth did provide a surprising result. While it was expected that sufficiently high concentrations of Cu would reduce growth rates of bacterial isolates, the level at which detrimental effects were observed was lower than initially envisaged (Chapter III; H4). Indeed, the lowest experimental concentrations of Cu in this study appeared to reduce the growth efficacy of the bacterial isolates growth. The mean concentrations of Cu at the LMS and TCS sites were 0.738 mM and 1.128 mM Cu, respectively (Chapter III); lethal concentrations of Cu, which completely inhibited all bacterial growth, were between 1 – 10 mM Cu. There is therefore the potential for Cu concentrations to completely inhibit bacteria growth at the TCS site, however the microcosm experiment conducted in this thesis employed a \log_{10} serial dilution of Cu, therefore further investigation at these concentrations would be required to determine if the lethal concentration of Cu is present at the TCS site. Furthermore, the effects of the root systems present at the TCS site would have to be taken account for in future examinations of the Cu toxicity of this site.

The LMS site was measured as having lower environmental concentrations of Cu (Chapter II and III). However, all bacterial isolates appeared to be affected by Cu equally regardless of the sample site used for sub-culture of the isolates. Inhabiting an environment in which the lethal concentration of Cu may be possible could affect both bacterial growth and diversity at the sample sites (Gordon et al., 1994), therefore may be a target for further investigation.

6.1.2.2. Temperature growth optima

As the temperatures measured at the critical zone were between c. 0 – 12 °C it was originally expected that psychrophilic as well as psychrotolerant species of bacteria would inhabit these regions. However, there was no evidence of psychrophilic bacteria found in this thesis (Chapter III). All bacteria were capable of sustained growth above the temperature (20°C) at which psychrophilic growth occurs (Morita, 1975). Conversely, the lower limits of growth were only achieved for three of the 35 isolates examined here. There was evidence of slow and persistent growth even at temperatures as low as 1°C (Chapter III). For bacteria to be growing they must therefore be metabolically active at these temperatures. This infers that while weathering and growth may be higher in the mid-summer season, it is expected that both of these activities will also occur during the mid-winter, more so for the TCS bacterial community as this site never reaches temperatures as low as 1°C.

6.1.2.3. The environmental effect on weathering

No statistical relationship could be made between weathering efficiency and taxa (Chapter III; H7), geographical sample site (Chapter III; H6), aerobic strategy or

temperature optima for growth and Cu tolerance. However, it was apparent that most bacterial isolates were capable of weathering mineral state phosphate (as indicated by MPS phenotype; Chapter III), exhibited a preferential temperature higher than that observed in the environment and were adversely affected by the presence of Cu, even at low concentrations. The optimal growth of the bacterial isolates studied here, was not afforded them from the environment from which they were sub-cultured. Consequently, the culturable bacterial community identified at LMS and TCS sites do not appear to be highly specialised to their environment since the environmental conditions measured here all have deleterious effects to bacterial growth regardless of the isolates geographical origins. This leads to the conclusion that the generalist MPS function, measured here, is not actively selected for within either sample site. However, as no isolate exhibited an optimisation for survival in this area, it could be hypothesised that this generalist MPS bacterial community are ubiquitous and that weathering capability merely provides a purchase within certain niche habitats to permit sustained growth.

6.1.2.4. Weathering function

If weathering is to offer a competitive advantage to bacteria in such an extreme environment then characterising the mechanism is important. It was anticipated that detection and isolation of the glucose dehydrogenase (*GDH*) gene could provide a method for rapid identification of one method of bacterial weathering phenotypes from soil samples *in-situ* (Chapter III; H5), yet the resulting PCR amplicons from the *GDH* gene assay were highly non-specific. The gene that was

apparent in the published literature (Shigematsu et al., 2005) bore no resemblance to any amplicon obtained in this study.

While it was possible that none of the bacterial isolates examined possessed the *GDH* gene, this was not believed to be probable due to the high occurrence of the MPS phenotype in an assay using glucose as the sole carbon source (Chapter III); however, this was an unqualified assumption. The phylogenetic similarity between the isolates studied here and those described by Shigematsu et al. (2005), on which the oligonucleotides were designed, combined with the high number of isolates exhibiting the MPS phenotype, results in the high likelihood of the isolates possessing the *GDH* gene. It is believed to be more likely that there was a lack of homology between primer sets and target genes or the cross hybridisation of other genes types within the assay and that this may have resulted in the failure to detect the *GDH* gene. Unfortunately the data obtained did not indicate why this method was a failure, only that no *GDH* gene was found. Although this attempt was not met with success, it is believed that this approach does still hold merit in determining the presence of bacterial-induced weathering, as organic acid production and the biochemical pathways employed are likely mechanisms for this MPS process.

While the production of gluconic acid is a process which involves the *GDH* gene as well as the consumption of glucose as a carbon source, this may be an oversimplification of the potential processes associated with organic acid production. The metabolic processes and products involved in glucose metabolism are numerous (Figure 6.1). Glucose as a substrate is capable of spawning metabolites

such as hexane, gluconic acid, sorbitol and α-cyanohydrins. Therefore another probable failing of this method may be that glucose is metabolised by a number of pathways and other metabolites, such as glucaric acid, are produced and these alternate metabolites may have resulted in the MPS phenotype recorded in chapter III, without the need for the *GDH* to be expressed or present within the cell.

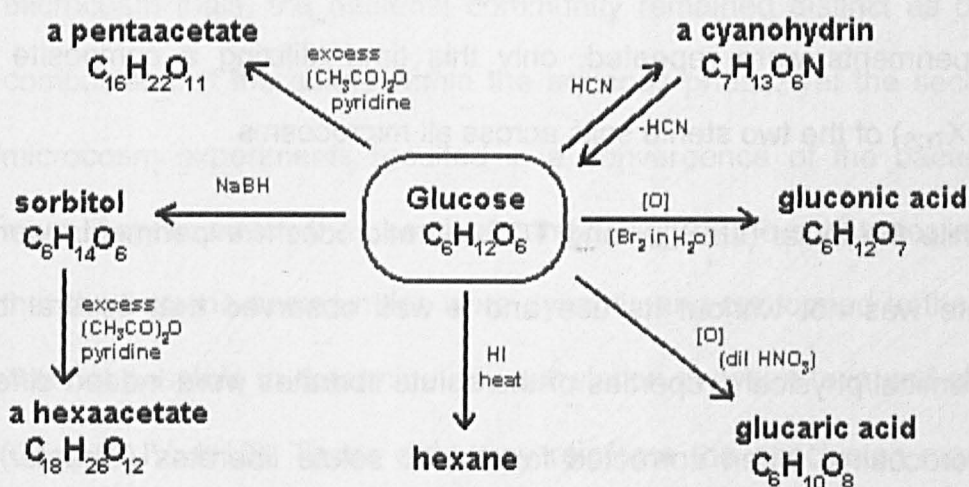


Figure 6.1: An example of several potential pathways and endproducts that can occur through the metabolism of glucose as a carbon source. While gluconic acid is one such product, it is not the only one.

6.1.3. Measuring the effects of bacteria on weathering *in-vitro*

Measuring bacterially-induced weathering in the field is not methodologically feasible, as although organic acids can be detected, their origin would be unknown; therefore microcosm experiments were initiated here (Chapter IV). These microcosm experiments were intended to correlate the abundance of individual bacteria taxon with increases in the solute liberates associated with

weathering of silicates. Initially, the critical zone soils from LMS and TCS sites were thought to be highly similar (Moulton & Berner, 1998, Moulton et al., 2000) which suggested that a direct comparison could be made between microcosms' solute liberates using LMS and TCS inocula. However, XRF and ICP-AES analyses of the soils (Chapter II and III), conducted concurrently with the initial LMS_{LMS} and TCS_{TCS} incubations, showed differences in the chemical compositions between the soils from LMS and TCS sites. To minimise the effects of the differences, while still investigating the sub-surface environment, the microcosm experiments were repeated, only this time utilizing a composite (MIX_{LMS} and MIX_{TCS}) of the two sterile soils across all microcosms.

While the initial (LMS_{LMS} and TCS_{TCS}) microcosm experiments were flawed, the data was not without its use and it was observed that several biological and chemical/physical properties of the solute liberates were indeed different between microcosms, when corrected for abiotic solute liberates (Chapter IV; H8). The bacterial communities measured were similar to their original environmental inocula (Chapter II). Also the some solutes were measured as being different in concentration between microcosms. This provided a measure of biotically induced weathering. Solute originating from the TCS_{TCS} microcosms indicated lower concentrations of Si, Cu, NO₃ and SO₄ as well as a lower pH than was measured from the LMS_{LMS} microcosms. Rather than increased concentrations of these solutes indicating weathering, it is possible that the lower concentrations of these solutes were consumed by increased bacterial activity (Chapter IV). This may be confirmed by monitoring the respiration rates of the microcosms, however this was not conducted on this occasion. Furthermore, the statistical power to this approach

was low as the original experiment design was not intended to utilize the abiotic controls in this manner, therefore confidence in the results is also low for all LMS_{LMS} and TCS_{TCS} experiments.

The second microcosm trials (MIX_{LMS} and MIX_{TCS}), using composite soil substrates also proved to yield unexpected results. Rather than the two differing inocula altering the substrate and subsequent solute liberates, it was apparent that the substrate had in fact altered the bacterial community (H8). From the first microcosm trials, the bacterial community remained distinct as did the chemical composition of the solute within the aqueous phase, yet the second trials of the microcosm experiments resulted in a convergence of the bacterial community, most likely to match the chemical composition of the substrates employed. Given that the bacteria communities were dynamic and conformed to the soil substrate it was not possible to determine any correlation between taxa and chemical species (Chapter IV; H10). These data may reinforce the conclusion made in chapter II that the presence of plants at the TCS sites may be altering the bacterial community and diversity through the provision of organics to the soils, as much of the original organics within the soils would have survived sterilization methods.

6.2. Conclusion

In conclusion, there appears to be a diverse bacterial community inhabiting the sub-surface critical zone (Chapter II; H1) and this diversity is likely to be influenced from root-forming plants at the soil surface (Chapter II; H3). The exudates and organics provided by the presence of a root-system appear to lower the diversity

of the bacterial community, resulting in a more specialised assemblage (Chapter II; H2).

The soil environment also plays a role in selecting for the bacterial composition (Chapter III; H6) as the temperatures and metal concentrations of the environment are not optimal for the growth of the bacterial community (Chapter III; H4) and may be exerting a selective pressure on bacterial communities. In addition, the bacterial functional role of weathering at the critical zone did not appear to be limited to a few specialized individual taxa (Chapter III; H5). Of the cultured isolates examined, 91% exhibited the MPS phenotype. Therefore, a generalist bacterial population are thought to contribute to the bacterial induced weathering at the critical zone, though the mechanism is not well understood (Chapter III; H7).

One interesting finding, was the predisposition for the bacterial community composition to alter based on the chemical composition of the substrate (Chapter IV; H8), rather than the substrate to alter under the influence of the bacterial community. This simple, and somewhat unexpected, event makes it very difficult to measure bacterial induced weathering *in-situ* as the bacterial community and the substrate will co-vary during the process of weathering and prising the two apart is problematic.

The final aspect of this thesis showed that over 90% of the bacterial community (Chapter V; H11), grown in microcosms with bacterial necromass as a carbon source (Chapter V; H12), utilized a heterotrophic survival strategy. Extrapolated to the Icelandic sample sites, this suggests that necromass may play a role in the development of a rich and diverse heterotrophic bacterial community in the

rhizosphere-poor soils. Although the inclusion of the bacterial necromass appeared to increase the abundance of *Alphaproteobacteria* in the microcosms, there was no distinction between LMS environmental bacterial communities and those measured experimentally in chapter V. Therefore, the oligotrophic environment of the LMS sample site would be expected to be capable of maintaining a substantial heterotrophic population.

6.3. Limitations to this study

The various methods used throughout this thesis have provided a detailed analysis of the sub-surface critical zone and the bacterial communities that inhabit this region. However, there are limitations to both the experimental approach and the methods employed that should be considered.

6.3.1. Unaddressed experimental issues and directions

Molecular methods are changing at a remarkable pace. Very soon the logistical and financial constraints on sequencing will be removed, permitting studies of millions of microbial cells from individual samples. However, regardless of how many sequences are obtained from a single sample it is not necessarily representative of the wider microbial community. Although the number of sub-samples and/or 16S rRNA sequences was sufficient for this study, the overall conclusions are largely confined to making deductions regarding this particular area of western Iceland.

Given another three years to study this topic, it would be advantageous to build on what has been discussed in this thesis. Expansion of the sampling area to include

other similar areas would permit more statistical emphasis on the results obtained and permit extrapolations to other geographical areas. Also the function of 'how' weathering occurs is still not clear. There was evidence of differing pH (Chapter II), generalist MPS capability (Chapter III) and heterotrophic behaviour (Chapter V), yet no conclusive evidence was obtained to state that the bacteria present at the sub-surface critical zone actually promote MPS efficacy through alteration of the local pH while employing a heterotrophic carbon strategy *in-situ*. These questions require a greater effort and a more in-depth investigation of the functional phenotype and genotype of the bacteria at the critical zone.

6.3.2 Presence does not equal function

With the advent of modern molecular methods of analysing and processing nucleic acids, molecular ecology has taken a leap forward in its ability to monitor and record the microbial world. This said, the measurement of microbial diversity, both in this thesis and the wider literature (Janssen, 2006, Lepleux et al., 2012, Ribeiro et al., 2011, Santelli et al., 2009, Whiteley et al., 2012), relies heavily on the detection of the 16S rRNA gene from the environment. Reliance on the 16S rRNA gene for measuring diversity can input a bias into studies as it provides more of a measure of diversity potential rather than actual diversity. The reason for this is that DNA fragments can persist in the environment following lysis of the original host cell (Nielsen et al., 2007) for thousands of years following cell death. These fragments, while likely degrading over time, may be PCR amplified alongside modern DNA and may be identified in studies even when no viable organisms of that type are present (DeSalle et al., 1992). This is an error inherent in many

studies, including this one. However, the land usage has not altered in the Skorradalur area of Iceland since the late fourteenth century, therefore the impact on the ecosystem would be limited (Vésteinsson, 1998).

6.3.3. Finding the right community balance

Comparisons of the bacterial community from the sub-surface critical zone were conducted for sites that harboured higher order plants, with a visible rhizosphere horizon (TCS 1 + 2), and sites harbouring lower order plants, showing little evidence of root formation (LMS 1 + 2; Chapter II). It was observed that the presence of higher order plants was related to increased concentrations of SOM at the critical zone and this organic-rich substrate had an adverse effect on the recovery of nucleic acids from the critical zone. This resulted in successfully processing only c. 50% of the samples from TCS sites. To abet this limitation of the methodology, a greater sampling effort was employed. As sampling effort is correlated to diversity measures (Magurran, 2004), the increased sampling effort was conducted both at the LMS and TCS sites. While this increase sampling effort ultimately yielded a sufficient number of samples for statistical analysis, the comparisons were unbalanced, with a greater number of samples from the LMS site. Comparisons therefore required the use of non-parametric analysis. This had the effect of lowering the power of the statistical analysis, though it was preferential to a balanced yet biased comparison of bacterial communities. In the cases where non-parametric analyses were conducted, the confidence in the results is lower than if a parametric test had been used as results are based on pooled data rather than examination of individual data points.

6.3.4. Community profiling only scratches the surface

The taxonomic resolution obtained by community profiling is generally poor and computationally complex, with few studies being able to identify bacterial taxa beyond the Class level (Nocker et al., 2007). In a bid to overcome this, NGS was employed to provide a larger database of sequences by which to infer a taxonomic identity to individual tRFs. Historically, clone libraries were used for this function and would consist of tens to hundreds of 16S rRNA gene sequences. However, NGS technologies can now provide researchers with 16S rRNA gene libraries of tens of thousands of sequences and ultimately permit greater confidence in, and taxa affiliated to, each tRF.

The use of NGS proved to have mixed success; for some abundant taxa it was possible to identify individual tRFs down to Family, or in a few cases Genus level, yet for the less well described (i.e. *Acidobacteria*) or abundant taxa, this thesis was still limited to Class level descriptions. Therefore, it was necessary to make all statistical comparisons of tRFs at the Class level where required, in order to maintain consistency. While examination of the lower taxonomic level may have provided a more detailed image of the sub-surface critical zone bacterial communities, this study was unable to provide that level of detail using NGS, and instead retained a consistent, yet broad, examination of the bacterial communities.

6.3.5. Tracing food webs

The use of stable isotopes as a measure of diversity was inherently flawed with respect to presence verses activity. An example of this can be seen from chapter V where the incorporation of isotopically labelled necromass was measured using

DNA-SIP. This method requires that the bacterial cells grow/divide and incorporate the isotopically-labelled carbon in the process. This method did not measure any organisms at the sub-surface critical zone that were metabolically viable but did not grow/divide. The viable bacterial community are therefore not accurately sampled using these methods as both viable non-growing individuals and those employing alternate sources of carbon would have been visualized in the lighter fractions of the experiment.

6.3.6. Phosphate weathering as a proxy for silicate weathering

The specific importance of silicate weathering has been discussed (Chapter I); however the experiments used in this thesis were capable of detecting weathering from a range of minerals, not merely silicates. For example, the weathering microcosm experiments (Chapter IV) measured solute liberates from a soil matrix that was silicate in origin. However, the confidence in the results of those experiments was low and therefore, most discussions of weathering capability are referenced back to the MPS phenotype results (Chapter III). In addition, the use of the MPS phenotype as a proxy for weathering capability permitted an examination of the phylogenetic relationship between bacteria and weathering that was not possible with the weathering data obtained from the microcosms experiments (Chapter IV).

Although the MPS data was valid, extrapolations to the natural environment are limited with regard to silicate weathering; for example, the MPS phenotype can only be used as a measure of mineral phosphate weathering. Silicate minerals, particularly basalt, vary in the chemical composition and concentrations of

phosphate contained therein. Drawing conclusions regarding the bacterial weathering efficacy in the environment using these data (Chapter III, MPS phenotype) must take account that the abundance of phosphate will not be uniform across different field sites. The bedrock from the sample sites examined in this study contained c. 3% PO_3 (Chapter III), but this can vary substantially in other locations (Flanagan, 1967).

However, although silicate weathering *per se* cannot be accurately measured in this thesis, the mechanisms believed to contribute to phosphate weathering, are also believed to contribute to silicate weathering. Hydrolytic processes are involved in both types of weathering (discussed in Chapter I) and are likely to result from lower pH conditions.

6.4. Future work to address research aims:

6.4.1. Determining the weathering mechanism

Future research directions would ideally include e.g. identification and quantification of the genes involved in the organic acid production, possibly through (Q)RT-PCR, to assess expression of the gene at different temperatures and metal concentrations. This will require a more physiological examination of the isolated bacteria than was conducted here. Monitoring of the carbon source (e.g. glucose) and correlating this to the measured production of organic acids, of which several are possible, is required prior to venturing to the molecular level of study described here.

Following a successful biochemical/physiological examination of the MPS phenotype it may be possible to identify functional genes that are correlated to the MPS efficacy. This would permit a link to be made between bacterial growth in the environment and the weathering capability of individual organisms. However, this approach should be limited to *in-situ* investigations. Any analyses conducted *in-vitro* is capable of identifying potential weathering agents, such as organic acids and siderophores without the need to depend on nucleic acid examination.

The PCR optimization performed in this study (section 3.3.8.) improved the *GDH* assay conditions; however non-specific bands were still apparent even at optimal temperatures, suggesting either a lack of homology between primer sets and target genes, or the cross hybridisation of other gene types within the assay. In order to clarify and improve these results, a primer set capable of isolating the *GDH* gene is needed. This can be achieved through the design of unique primers based on the nucleic composition of this gene. However as stated in chapter III, glucose is a carbon source for several metabolites other than gluconic acid. Any attempt to further this aspect of the thesis should broaden the scope to monitor the metabolites produced from the pathways used to incorporate the glucose. This will offer a more realistic understanding of the weathering process, rather than a simple examination of what does or does not metabolise glucose to gluconic acid.

Another aspect of interest for microbial ecology at the critical zone would be the question of function, or "who is doing what". The bacterial isolate AW1 11 (Discussed in Chapter III) was used as an example in some of the studies, as it exhibited psychrotolerant growth. It is also noteworthy that it was one of only three

isolates that did not display the MPS phenotype. This bacterium is able to grow well in cold conditions and persist in the critical zone, yet it does not tolerate high Cu concentrations and contributes nothing to the community through weathering. It is not known whether this bacterium fulfils a different function, or whether it employs a pseudo-parasitical relationship with the community. The fact that its role is unknown suggests that further research is required to investigate the persistence and function of this bacterium, and others like it, at the critical zone.

6.4.2. Weathering: a general microbial function

The microcosm experiments discussed in chapter IV were conducted on biologically closed systems and show a snapshot of the bacterial communities and the chemical composition of the water. In order to fully understand which bacteria influence the weathering of soils, a larger number of microcosms would allow destructive sampling in order to establish changes over time. If future analyses are carried out as per the methods in chapter IV on the water column and the soil matrix, cause and effect relationships between solutes and bacterial taxa could perhaps be established. Using this approach it may ultimately be possible to determine if there is any relationship between individual bacterial groups and chemical species liberation.

One question that was not addressed in this study was the role of soil fungal communities. The biologically active samples may have contained dormant fungal spores, which may provide answers to some of the questions raised in this thesis, as fungi are known to be prevalent in areas with root forming plants and capable of organic acid production.

6.4.3. The use of RNA expression rather than DNA

Future use of bacterial necromass within SIP should concentrate on the initial stages of incorporation of the isotopic label to ensure that the resolution of data is sufficient to answer any questions being examined and logistics permitting. Furthermore, the use of RNA-SIP may yield a more representative data set. As the RNA is a measure of bacterial activity rather than growth.

The detection of an organism or even a functional gene from the DNA obtained from environmental samples does not necessarily permit a measure of the functional role of an individual. It is possible for microbes to possess a functional gene, but not actively express it. This can be overcome by detection of mRNA in the environment as only functionally active microbes will actively express these nucleic acids. However some researchers argue that the differences observed between RNA- and DNA-based measures, while significant, offer little in the way of understanding the microbial community (Duineveld et al., 2001, Nogales et al., 2002).

Extraction of nucleic acids from andosols

i.1. Background

Several of the chapters in this thesis utilize the same method of nucleic acid extraction. The details addressed in this appendix serve to justify the choice of nucleic acid extraction protocol employed.

The initial stages of this research were primarily centred on the extraction and purification of total nucleic acids for critical zone soil samples. However, the progress of the study was hindered by the unpredictable quality and quantity of the nucleic acids obtained. During the experiment design and budgeting, the Mobio Powermax™ soil DNA isolation kit was chosen as the sole method for DNA extraction. However during subsequent examination of nucleic acids it was apparent that the majority of samples failed to yield any DNA suitable for PCR amplification.

In order to ensure that sufficient quantity and quality of nucleic acids were extracted and that this provided a representative and repeatable measure of the bacteria present in the sub-surface soils examined in this study, several extraction protocols were investigated. Two of these methods were commercially available extraction kits, while the other extraction methods covered were variations on protocols available within the literature.

i.2. Protocols used in trial:

CTAB/EDTA nucleic acid extraction,

FastDNA® spin kit for soil,

Mobio PowerMax® soil DNA isolation kit,

PHE:CHL:IAA total nucleic acid extraction,

Cell separation using Histodenz™ gradient buffer,

All nucleic acids were visualized on a 1% (w/vol) agarose gel with ethidium bromide staining to confirm the extraction of nucleic acids.

i.3. Extraction results

The results of this trial showed that the Phe:Chl:laa bead beating described by (Griffiths et al., 2000) gave the most reliable extractions (Table i.1). However with only a 50% success rate there was still the potential for not achieving a fully representative picture of the bacterial community at the critical zone, as it was likely that only samples rich in biomass and low in extraction inhibitors such as metals and humics would yield sufficient nucleic acids suitable for PCR amplification.

Attempts were made to improve the Phe:Chl:laa bead beating extraction method by seeding with salmon sperm prior to extraction. The eukaryotic nucleic acid was intended to bind to and saturate clay particles and humic acids. These have the potential to bind to prokaryotic nucleic acid, preventing successful extraction of prokaryote nucleic acids into the aqueous phase. While a pilot of this approach suggested that there was improved consistency in extraction efficacy, this approach was abandoned as the effect of eukaryotic nucleic acid on bacterial

contamination and PCR efficacy was unknown.

Table I.1: Showing the success rate of the extraction of nucleic acid from critical zone soils for each extraction method trialled.

Extraction method	CTAB*	PowerMax	Phe:Chl:laa	FastDNA	Histodenz™
% Success	45.80%	20%	50%	22.20%	0%
<i>n</i>	54	8	54	54	8

* CTAB method produced good results only for site with lower order plant coverage, higher order plant covered samples yielded no nucleic acid.

I.4 Conclusions

The use of commercial extraction kits did not provide adequate nucleic acids to permit the study of bacterial diversity at the critical zone. Cell separation using Histodenz has been successful in previous applications for the separation of cells from the soil matrix, however in this case there was no evidence of cell separation from critical zone soils.

There was some success using the CTAB/EDTA extraction methods, however this was limited to samples originating from the LMS sample sites. This method of extracting nucleic acid would not permit any comparison between TCS and LMS critical zone bacterial communities. The most consistent method of nucleic acid extraction was the Phe:Chl:laa extraction. Not only did this method produce the highest success rate, it was suitable for use on soils from both the LMS and TCS sites. This said, there was a greater success rate at the LMS site than at the TCS

site (2:1) resulting in an unbalanced experimental design. The samples that had been collected were insufficient to compare TCS and LMS bacterial communities when it was calculated that ~50% would not provide any nucleic acid for examination. To overcome this, the experiment design was altered to increase sampling effort and statistical comparisons would be made using non-parametric methods to account for the unbalanced nature of the samples.

i.5. Appendix detailed methods

i.5.1. CTAB/EDTA 16S rRNA gene extraction

Nucleic acids were extracted from 0.5 g (wet weight) of soil in a 2 mL Eppendorf tube. To this up to 1.5 mL of 20 mM EDTA was added and the tube shaken at 100 rpm for 1 hour. Once complete the tube was centrifuged at 16,000 xg for ten minutes and supernatant discarded. To the soil pellet, 500 μ L of lysis buffer (0.5% SDS, 25 mM EDTA and 20 μ g mL⁻¹ proteinase K) was added and mixed by inversion. A freeze thaw element was introduced by freezing the tube at -20°C overnight. The tube was permitted to defrost at room temperature before being incubated in a water bath for 25 minutes at 55°C; mixing by inversion every ten minutes. One hundred μ L of 5 M NaCl and 80 μ L of 10% CTAB, both preheated to 65°C, were added to the tube prior to inversion followed by a further ten minutes incubation at 65°C. Following incubation, 680 μ L of chloroform:isoamyl alcohol (Chl:laa; 24:1) was added to the tube and shaken to form an emulsion. The tube was centrifuged at 16,000 xg for five minutes to separate aqueous layer within tube. This aqueous layer (approx. 600 μ L) was removed and placed into a clean Eppendorf tube and 360 μ L of isopropanol was added and mixed by inversion. The

tube was left at room temperature for at least 2 hours for nucleic acid to precipitate. The tube was centrifuged at 16,000 xg for ten minutes to pellet nucleic acids, and then supernatant disposed of. Nucleic acid pellets were washed in ice cold 70% (vol/vol) EtOH to remove residual salts from pellet, and then EtOH removed and discarded. After pellet was air dried it was resuspended in 50 µL Tris-EDTA (pH 8) buffer.

1.5.2. FastDNA® SPIN kit for soil

All samples processed using this commercial kit was done as per manufacturers' instructions.

1.5.3. Phe:Chl:laa total nucleic acid extraction

This extraction protocol is a minor variation from Griffiths et al (2000). Soil samples of 0.5 g (wet weight) were added to lysis matrix E tubes (qbiogene, 6914-100) with 500 µL of phosphate buffer added (120 mM K₂PO₄, pH 8.0, with 5% CTAB) and 500 µL of Phe:Chl:laa (25:24:1). Tubes were shaken at 5,500 rpm for 30 seconds, and then transferred directly to an ice bath. The next stage was to separate aqueous phase by centrifuging tube at 10,000 xg at 4°C for 5 minutes, then transferring supernatant to a clean tube. In order to remove any residual phenol from supernatant it was washed in 500 µL of Chl:laa, (24:1) and inverted to mix. The supernatant was removed and added to a clean tube containing 500 µL of polyethylene glycol (PEG) solution (1.6 M NaCl and 30% PEG 6000); higher volumes of PEG were used if supernatant was brown in colour, indicating high humic content. This was left overnight at 4°C to precipitate. Following precipitation, the tubes were centrifuged at 14,000 xg at 4°C for ten minutes to pellet nucleic

acid. This pellet was then washed in ice cold 70% (vol/vol) EtOH to remove salts. The EtOH was removed and pellet allowed to air dry before resuspension in 50 μ L of Tris-EDTA (pH 8).

i.5.4. PowerMax® Soil DNA isolation kit

Samples were processed according to manufacturers' instructions with one alteration. The volume of elution buffer was reduced to 1 mL to concentrate the elution of nucleic acids obtained.

i.5.5. Cell separation using Histodenz™

A cell extraction method using a gradient forming solution Histodenz™ was attempted; this was closely based on the protocol described by (Courtois et al., 2001). Here was the only indirect extraction method examined in this project. Five g (wet weight) of soil was suspended in 0.9% NaCl up to 50 mL and four 4 mm glass beads added. This tube was vortexed for 90 seconds to homogenise the soil into slurry. Into a 50 mL falcon centrifuge tube 20 mL of slurry was placed and 7ml of Histodenz™ (1.3 g mL^{-1}) was injected underneath the slurry using a pipette to form a Histodenz™ cushion, then centrifuged at 10,000 xg for 30 minutes. The transition layer between supernatant and Histodenz™ cushion, containing cells removed from soil, was extracted and transferred to a clean Eppendorf tube prior to cells being pelleted and washed in sdH_2O to remove any trace of the Histodenz™ from cells. All cells were then subjected to the CTAB/EDTA extraction (Ref i.5.1.) to extract nucleic acids prior to any downstream application.

Appendix ii

Soil homogeneity effects on nucleic acid extraction yields

ii.1. Background

During the course of this research, it was observed that the quantity and quality of nucleic acids extracted from soil samples was low using the (Griffiths et al., 2000) extraction protocol. Several different protocols and commercial kits were employed in an attempt to improve this (appendix i), yet none proved as effective. The next step was to attempt an optimization of this protocol.

A discussion with colleagues regarding this issue raised the hypothesis that the homogeneity of the soil samples may be a factor which resulted in the poor and inconsistent results to date. Two possible solutions were postulated:

- 1) Multiple extractions could be performed and elutions pooled
- 2) Homogenization of soils prior to extraction

ii.2. Extraction and quantification.

Samples of critical zone soil were selected: these consisted of LMS (143, 152) and TCS (343, 352) samples. Aliquots of 10 g (wet weight) were placed into four separate 50 mL falcon tubes (Fisher Scientific, UK) to this was added 10 mL of sterile 1x PBS solution. Each of the four tubes was inverted resulting in a soil slurry and each tube had 500 μ L of soil slurry removed using a pipette and placed into a lysis tube; this was repeated to provide six replicates for each sample.

Following the harvesting of heterogeneous soil slurries, to each of the 50ml falcon tubes were added four 4 mm sterile glass beads (Fisher Scientific, UK) and the

tubes vortexed for 60 seconds to homogenise the soil slurry. Removal of the soil slurry was repeated as before to provide a further 24 extractions. The extractions were carried out as per the methods described by (Griffiths et al., 2000), with an alteration of the bead beating stage of the protocol. Three replicates had nucleic acids extracted exactly as per the protocol; however, three replicates, from both homogenous and heterogeneous slurries, were subjected to three cycles of bead beating with the aqueous layer being removed after each bead beating cycle and replaced with 500 µL of sterile phosphate buffer. Replicates with multiple bead beating stages had aqueous phases pooled within samples to attempt to increase yield for each sample. The resulting extractions were stored at -20°C.

Quantification of extractions was carried out using Sybr® green quantification methods (Method: ii1) and the results plotted against a calibration curve to obtain approximate yield quantity. The replicates subjected to three bead beating cycles were quantified but no further analysis of samples was carried out.

ii.3. PCR amplification and tRFLP analysis.

All 24 remaining samples were amplified for tRFLP analysis, using standard universal bacterial 63f-530r primers using conditions described within Chapter II. All products from amplification were verified on a 1.25% agarose gel prior to tRFLP analysis being conducted.

Comparisons of the allele reports for each of the soil samples were compared using a Chi-Squared goodness of fit test to test for differences between the tRFs detected for heterogeneous and homogeneous extraction protocols.

ii.4. Results

The quantification of all 48 extractions indicated that 11 of the 48 extractions yielded a measurable quantity of nucleic acid (Fig ii.1) and all were from site 152. The signals measured for the remaining samples appear to be beyond the lower detection limits of the quantification method.

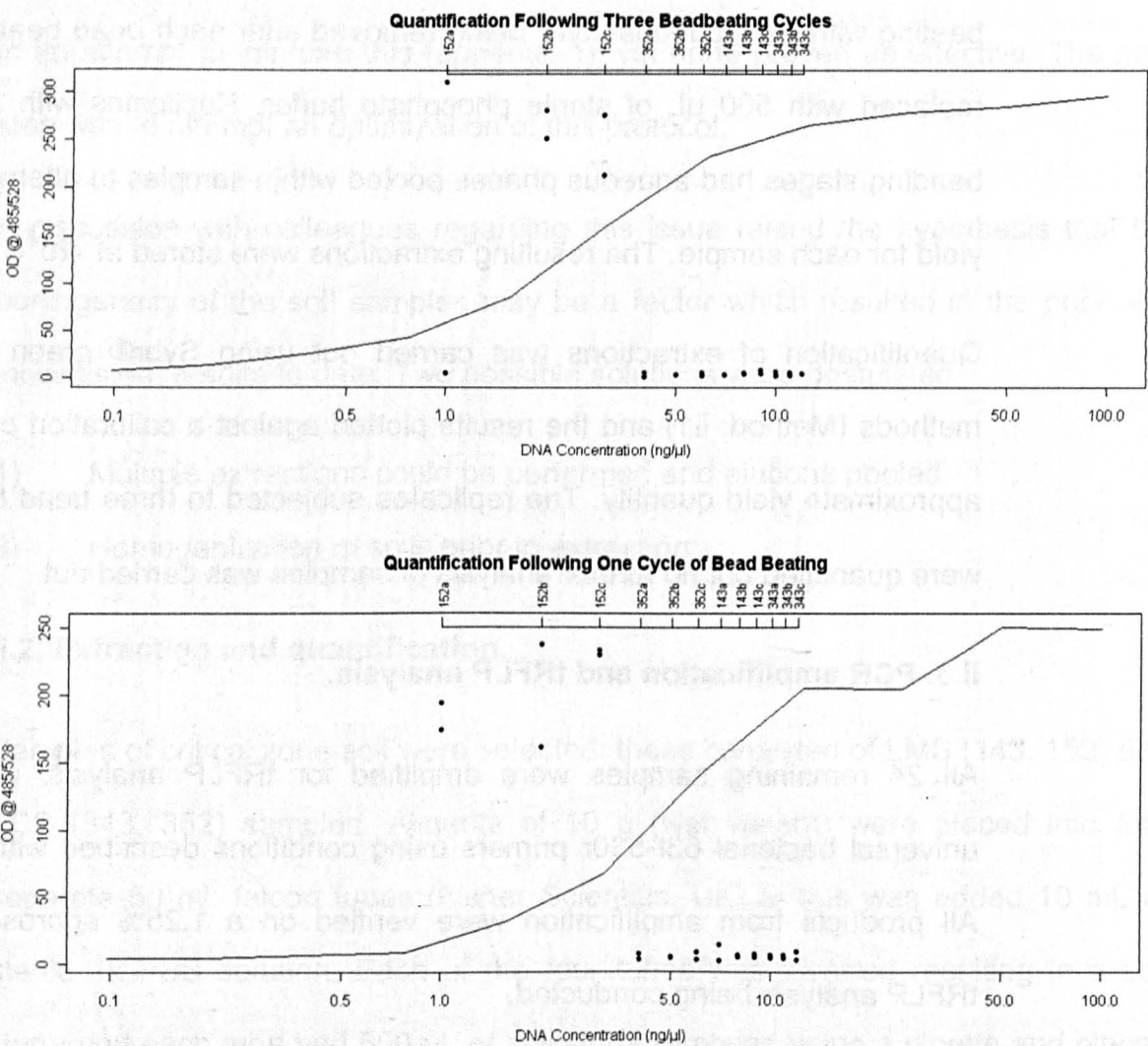


Figure ii.1: The log concentration of calibration curves are displayed with points showing each extraction yield. Reading horizontally from each point to the calibration curve it is evident that only extractions from site 152 yield concentration clearly above zero. All other samples are at lower detection limits of the method.

Points in blue are from homogenized extraction and points in red are heterogeneous extractions.

Amplicons formed during tRFLP amplification were displayed on an agarose gel (Figure ii.2) and showed that only samples, both heterogeneous and homogenized, from site 152 resulted in any significant amplification. The heterogeneous extraction of 143c shows a very weak product on the gel image.

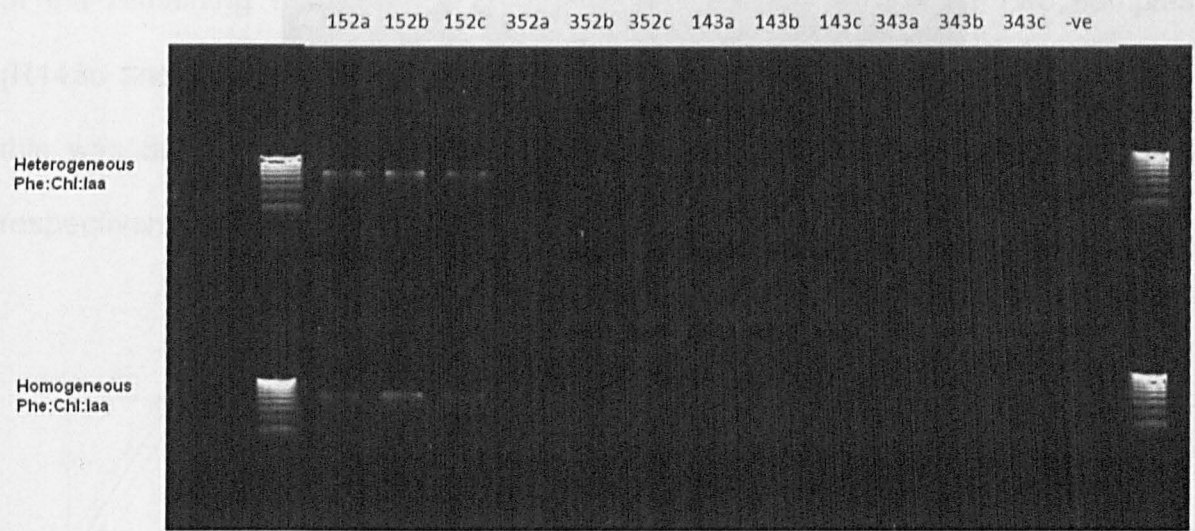


Figure ii.2: Amplicons from 63f-530r tRFLP PCR protocol displayed on a 1.25% agarose gel. Only seven bands are visible out of the 24 reactions. (Hyperladder IV used).

The electropherograms analysed using Genemarker software show that while the samples from site 152 display a diverse community the dominant tRF peaks are evident in most of the samples processed. The fragment gel image (Figure ii.3)

showed the distribution of tRF peaks and two distinct tRFs (52 n.t. and 155 n.t.) are readily seen in most samples.

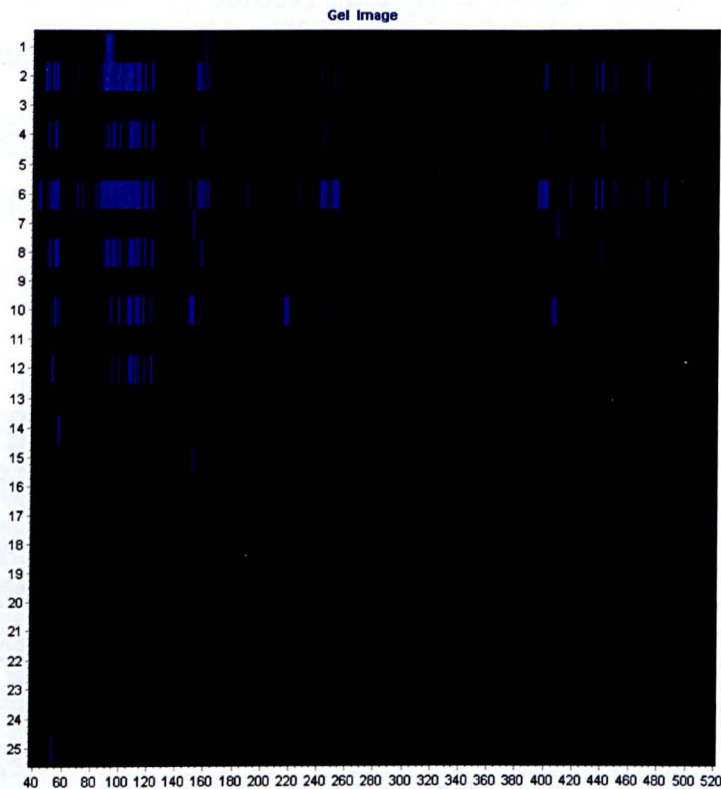


Figure ii.3: The extractions from site 152 show a diverse community, however the remaining samples also display some of the more common allele peaks; indicating that the yield from the extractions was above zero.

Lane identification:

1-H352a, 2-R152a, 3-R143a, 4-H152a, 5-H143a, 6-R152b, 7-R143b, 8-H152b, 9-H143b, 10-R152c, 11-blank, 12-H152c, 13-H143c, 14-R352a, 15-R343a, 16-H343a, 17-R352b, 18-R343b, 19-H352b, 20-H343b, 21-R352c, 22-R343c, 23-H352c, 24-H343c, 25-R143c.

R = heterogeneous extraction.

H = homogenised extraction.

Although the gel image showed the presence of allele peaks in most samples, the background noise in the samples resulted in peaks being indistinguishable. As a result, the NMDS ordination grouped 18 of the 24 samples at the same region and presented the site 152 extractions as a separate group outside of main body (Figure ii.4). The ordination clearly highlighted the dissimilarity of the six extractions from site 152 (stress, 0.13); however this was relative to the similarity of the remaining samples that yielded no significant amplicons. Two samples (R143c and H352a) appeared outside of the main body of failed amplifications; this was due to a single peak being evident at the 52nd and 90th n.t. location respectively on the electropherograms.

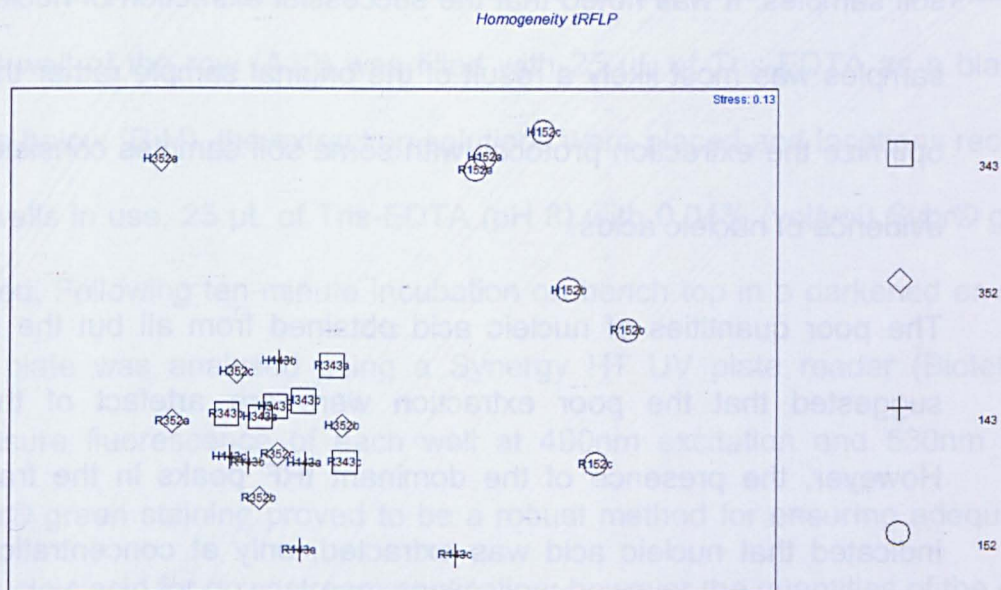


Figure ii.4: NMDS of all samples. Samples originating from site 152 are clearly distinct from the main body of the ordination. Those samples that presented poor extractions and failed amplification are present in lower left corner, showing little variability or pattern relating to site or extraction method.

By performing a Chi Squared test on the pooled allele peak data from all six extractions from site 152 it was shown that there was no significant difference between the heterogenous and homogeneous soil slurry methods of extraction ($p > 0.05$). Homogenization did not alter the community of bacteria detectable using tRFLP analysis.

ii.5. Conclusions

It was evident that homogenization of the soil slurries had no significant effect on the yield or the diversity of the nucleic acids that were extracted from critical zone soil samples. It was noted that the successful extraction of nucleic acids from soil samples was most likely a result of the original sample rather than the attempt to optimize the extraction protocol; with some soil samples consistently yielding little evidence of nucleic acids.

The poor quantities of nucleic acid obtained from all but the site 152 samples suggested that the poor extraction were are artefact of the soil substrate. However, the presence of the dominant tRF peaks in the fragment gel image indicated that nucleic acid was extracted, only at concentrations too low to be experimentally viable.

ii.6. Appendix detailed methods:

Sybr® green quantification of nucleic acid extractions.

In order to ascertain the quantity of nucleic acid yielded from an extraction prior to use it is necessary to quantify the elution. The use of Nanodrop on nucleic acid extractions from soils has been used widely, however this requires that the extractions are purified prior to analysis or that the extraction protocol or kit used has a method of removing humics and organics from the elution prior to analysis. This can be costly for larger scale projects or samples in which the nucleic acid yield may be compromised by the efficacy of the soils to extraction.

A logistically inexpensive method of quantification was to analyse extractions using Sybr® green stained nucleic acids on a UV plate reader. 100 ng μL^{-1} of salmon sperm DNA was double serially diluted with Tris-EDTA (pH 8) until 0.09 ng μL^{-1} of salmon sperm was achieved. From resulting Salmon sperm DNA dilutions, 25 μL was pipetted onto the top row of a clear flat bottomed 96 well plate and the final well of the row (A12) was filled with 25 μL of Tris-EDTA as a blank. In the rows below (B-H), the extraction solutions were placed and locations recorded. To all wells in use, 25 μL of Tris-EDTA (pH 8) with 0.04% (vol/vol) Sybr® green was added. Following ten minute incubation on bench top in a darkened environment, the plate was analysed using a Synergy HT UV plate reader (Biotek, UK) to measure fluorescence of each well at 490nm excitation and 530nm emission. Sybr® green staining proved to be a robust method for ensuring adequate yields of nucleic acid for downstream application; however the quantities of the extraction product required proved great. With half of all extraction yield used in the quantification stage there is little remaining for further analysis and any required optimization of applications.

Appendix iii

Oligonucleotides (primers) used in this thesis

Table iii.1: Details of the primers used in this thesis and locations of their usage.

Primer	Sequence	Reference	Chapter(s):	Target gene
63f	5'-CAGGCCTAACACATGCAAGTC-3'	(Marchesi et al., 1998)	II and III	16S rRNA gene
530r	5'-GTATTACCGCGGCTGCTG-3'	(Muyzer et al., 1993)	II and III	16S rRNA gene
27f	5'-AGAGTTTGATCCTGGCTCAG-3'	(Lane, 1991)	III	16S rRNA gene
1541r	5'-AAGGAGGTGATCCAGCCGCA-3'	(Embley, 1991)	III	16S rRNA gene
GDH-f1	5'-TGGAGCTACCAGACCGTCCA-3'	(Shigematsu et al., 2005)	III	GDH Glucose dehydrogenase gene
GDH-r1	5'-GGCAGGCGGTCCTGCCACAG-3'	(Shigematsu et al., 2005)	III	GDH Glucose dehydrogenase gene

16SrRNA gene sequences

Table iv.1: The accession numbers for the sequences recorded throughout this thesis are given below.

Method	Accession numbers	Database	Chapter
454	4505422.3 - 4505424.3	MG-Rast server	II
454	4505426.3 - 4505428.3	MG-Rast server	II
454	4505425.3	MG-Rast server	IV
454	4505429.3	MG-Rast server	IV
Sanger	KC164992 – KC165026	NCBI server	III

Supplementary results Chapter V

Rationale:

Further data than presented on the DNA-SIP probing in chapter V is available here. The rationale behind this is that the data here was not of suitable quality to be included in chapter V.

Phospholipid fatty acid stable-isotope probing (PLFA-SIP) was used in addition to the DNA-SIP to determine the individual taxonomic groups (Eukarya, Archaea, Bacteria) that were being isotopically enriched in the microbial community.

Results

PLFA analyses showed that ^{13}C was incorporated in to microbial lipids < 24 hours after exposure to labelled necromass. Lipids 16:0 and 18:1 (b) showed the greatest incorporation of the label (Figure v.1). The closest taxonomic match to these biomarkers was for the domain Bacteria. Of the 23 lipid biomarkers identified, 12 were shown to have incorporated carbon from necrotic bacterial matter. Eleven biomarkers (c. 48%) were identified through GC-MS yet no incorporation of the label was measured by GC-C-IRMS.

This method of monitoring the isotopic label had the ability to simultaneously record several taxonomic groups, over multiple kingdoms. However the resolution of this method did not permit identification of the individual groups to the same level as DNA-SIP. Discussing individual PLFA biomarkers in a taxonomic manner was ambiguous in this instance as several published articles attribute differing

nomenclature to similar biomarkers (Bossio & Scow, 1998, Moore-Kucera & Dick, 2008, Pratt et al., 2012, Vestal & White, 1989). The institution that performed the analysis did not use any standards when processing samples, therefore any results obtained here cannot be compared to other studies nor affiliated to any taxa with confidence. The two PL showing the highest $\delta^{13}\text{C mL}^{-1}$ were 16:1 and 18:1(b); both are similar to other PL originating from the domain Bacteria.

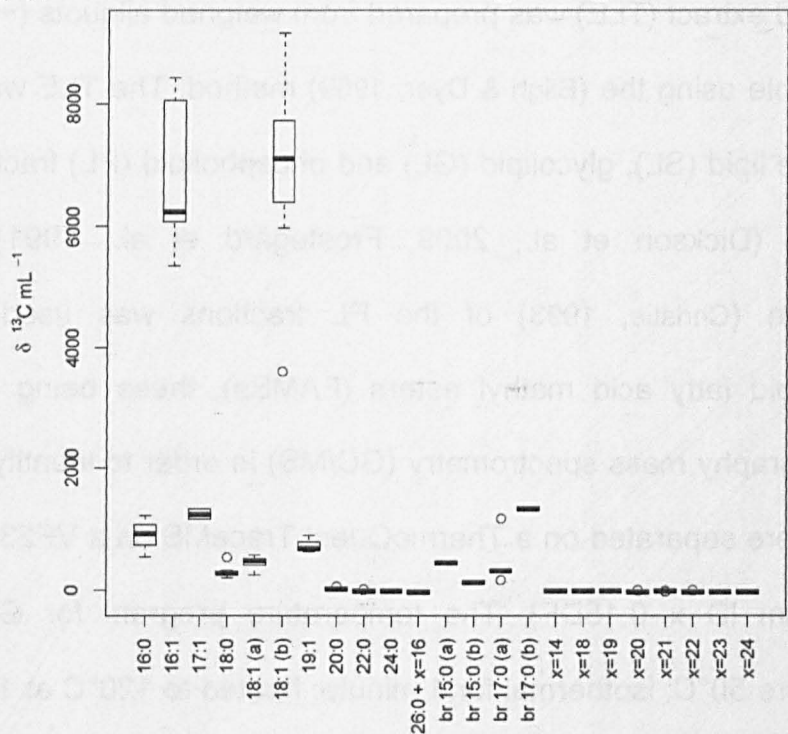


Figure v.1: Box and whisker plot showing the median $\delta^{13}\text{C mL}^{-1}$ of each biomarker for eight microcosm samples taken throughout time series. Control not included. PLs identified by $x = \#$, refer to methyl esters of di-carboxylic acids with the number of methylene groups identified.

Detailed methods:

Phospholipid fatty acid analysis

As isotopically labelled necromass can be incorporated into cellular structures other than DNA, the incorporation of the label into cell lipids was examined. Nine

isotopically labelled soil aliquots were examined for ^{13}C enrichment by PLFA analysis. This was conducted on location at the University of Bristol. One sample was used for background measurements, having no isotopic label present; a further eight samples (21, 45, 48, 51, 69, 75, 216 and 360 hours) were examined for ^{12}C : ^{13}C ratio.

A total lipid extract (TLE) was prepared from weighed aliquots (~2 g, dry weight) of each sample using the (Bligh & Dyer, 1959) method. The TLE was then separated into simple lipid (SL), glycolipid (GL) and phospholipid (PL) fractions as described previously (Dickson et al., 2009, Frostegård et al., 1991). Acid catalysed methylation (Christie, 1993) of the PL fractions was used to produce the phospholipid fatty acid methyl esters (FAMES), these being examined by gas chromatography mass spectrometry (GC/MS) in order to identify compounds. The FAMES were separated on a ThermoQuest TraceMS on a VF23-ms column (60 m x 0.32 mm ID x 0.15DF). The temperature program for GC/MS was: initial temperature 50°C; isothermal for 1 minute; heated to 120°C at 15°C minute⁻¹; then 250°C at 3°C minute⁻¹; then isothermal for 15 minutes. Samples of 1 µL were injected splitless.

Values for $\delta^{13}\text{C}$ mL⁻¹ were measured for identified compounds by gas chromatography combustion isotope ratio mass spectrometry (GC/C/IRMS). The method involved separation of FAMES on a VF23-ms (60 m x 0.32 mm ID x 0.15DF) column using a Delta V plus instrument (Thermoscientific, Essex, UK) with the following temperatures: initial temperature 50°C; isothermal for 2 minutes;

heat to 100°C at 10°C minute⁻¹; then 250°C at 4°C minute⁻¹; then 15 minutes isothermal. The SL and GL fractions were not analysed.

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